

Britain reviews its defence

THE results of the British defence review initiated after the February election will soon be published and now, with Labour safely returned, they are likely to be received favourably by the government. Already political vultures are surrounding the Ministry of Defence and pointing to what they could achieve if handed a tiny fraction of the money allotted for defence; the impressive list runs from better hospitals to more performances of operas. The snag is that it is most unlikely that defence spending will be trimmed by any significant amount and certainly not by the £1,000 million, or 30%, that the left wing has been demanding.

The early talk, and indeed that enshrined in Labour's recent election manifesto, was of bringing Britain's defence expenditure into line with that of its western European partners. Later in the election campaign, perhaps when the contents of the review had been made known to Labour politicians, the talk was more mutedly of not expanding expenditure and of carefully reconsidering overseas commitments when they come up for review. An uninformed guess, then, is that the review, far from revealing large areas of fat to be trimmed off, will simply say that Britain must be more modest in future. For the statistics on European comparisons show clearly that to talk of bringing expenditure into line with that of, say, France and Germany depends very much on what criterion one uses for being 'in line', and by one criterion, at least, Britain is irreproachably in step. The accompanying table provides some relevant figures on military spending in Western Europe.

Since 1950, military spending by European countries has grown relatively slowly, once inflation has been accounted for. Indeed in the years 1949-68 Britain's military expenditure grew on average by only 2.2% per year. France, with its nuclear development in the 1950s, showed a higher rate of 5% per year and West Germany,

re-arming, grew by 6% per year. But all three countries had, by the late 1960s, stabilised their expenditure, and defence, in real terms, was hardly growing at all. As the table shows, growth subsequently re-established itself; only France has managed to keep things in check. Not too much, however, should be made of this recent growth, which may well reflect the difficulty of preventing military costs from rising more rapidly than other costs in inflationary times rather than any deliberately expansive policy.

What is evident from the table is the serious weakness that a depressed gross national product (GNP) imposes on a country with global and nuclear pretensions. In other ways Britain's expenditure looks quite lean and there is no conscription, by contrast with France and Germany, not only to put up the expense of the forces but to pull young men out of productive (and taxpaying) occupations. It is the percentage-of-GNP indicator that makes Britain seem so far out of line; there are few other countries in the world which spend as much of their GNP on defence.

The question is thus not one of how to save a few million pounds here and there with small scale economies. It is much more fundamentally, should Britain go on spending defence money at the same *per capita* rate as her near neighbours, thereby providing highly sophisticated armaments for her forces, or should she recognise her economic limitations and aim to bring the figure of 4.9% down to nearer, say 3.5%?

Presumably major surgery, such as the elimination of Britain's nuclear capacity, is the only way that such a saving could be achieved in the next ten years, and this is not an option that is likely to have been considered seriously. This is a shame; the rationale for possessing nuclear weapons is not so strong that it could not profit by occasional exposure and criticism if only to ensure that a new generation, to whom CND is only an historical episode, is apprised of the disadvantages, as well as the supposed advantages of the possession of nuclear weapons. It would be unfortunate if these items of mass destruction came to be forgotten about by the public and thus subject to the pressures only of technological momentum and of those employed in their construction.

Defence statistics for Britain, France and West Germany in 1973

	Population (million)	GNP (\$1000 million)	Inflation 1969-73	Military expenditure				Armed forces		R & D as % of total expendi- ture
				\$ million	Growth 1969-73†	% of GNP	Per capita \$	(thousands)	% of all men aged 18-45	
Britain	56	177	7.7	8,673	10.9	4.9	155	352	3.4	9.0
France	52	277	6.0	8,438	7.0	3.1	162	504	4.9	9.0
West Germany	62	385	5.2	11,291	9.9	2.9	182	475	4.0	5.1

† Not corrected for inflation

Sources: *The Military Balance* 1974-1975 (International Institute for Strategic Studies.); *SIPRI Yearbook* 1973.

Be warned that all comparisons of military expenditure suffer from variations in definitions. Thus this table should be seen as no more than a general indication of comparisons. See page 102 of *The Military Balance* for more detail.



Agriculture and sunspots

WORLD food reserves have, until recently, been larger than the variability in production associated with the weather, and oscillations about the general upward trend of agricultural productivity could be ignored. It is now apparent, however, that urgent consideration has to be given to the impact of climatic changes on food production. The world's grain reserves have dwindled from the 1969 peak (19% of annual consumption) to only 7% of annual consumption, less than the variability of about 10% induced by the weather. Although many people have reported associations between the solar cycle and the weather, relatively little attention has been paid to the fact that the 11-year and the 22-year sunspot cycles could produce important modulations of agricultural productivity.

The world wheat production figures for 1949-73 can be used to show how the sunspot cycles appear to influence food production. Sunspot maxima occurred at the end of 1957 and again in 1968 and it seems significant that global wheat production in 1958 was greater than in each of the next five years, whereas that in 1968 was greater than the average of the next four years. In 1954 (a year of sunspot minimum) production was less than in any of the two preceding or the two following years. In North and Central America, for example, the crop in 1954 was smaller than any other year for which we have data; the average crop during the five previous years was 25% bigger than that of 1954.

The fact that wheat production in the Northern Hemisphere increases at around sunspot maximum can be deduced from the wheat figures for many countries. In the People's Republic of China, the annual production during 1956-58 (centred on the 1957 sunspot maximum) was 22% greater than during the period 1960-65; the total produced in 1958 has not been equalled in any year up to and including 1973. The average production in the Soviet Union during 1956-58 was, as in China, greater than during 1960-65; in 1958 the crop was 54% bigger than in 1963. The average wheat production in Canada during 1967-69 (centred on the 1968 sunspot maximum) was 27% greater than that during the four subsequent years, 1970-73; production in both 1968 and 1969 was higher than in any of the four years 1970-73.

Over much of the Northern Hemisphere, therefore, wheat production appears to be significantly enhanced near sunspot maximum and reduced at sunspot minimum. In parts of the

Southern Hemisphere, however, the opposite is observed. In Argentina, for example, the three years when most wheat was produced were 1954, 1964 (the two sunspot minimum years) and 1963; the average crop for these three years was 50% greater than for the nine years 1965-73. The African wheat crop in 1954 was bigger than in any of the next eight years; it was thus, as in Argentina, bigger than in any year until 1963. In Australia the 1957 (sunspot maximum) crop was smaller than

Researchers at the Appleton Laboratory, in England, say that the present low level of world food reserves may be associated with the decline in solar activity during the approach to the 1974-75 sunspot minimum. Presumably because of their effects on temperature and rainfall, the 11-year and 22-year sunspot cycles appear to influence agricultural productivity in certain parts of the world. Modulations of 10% to 50% of the wheat production in China, the United States and the Soviet Union seem to be correlated with the solar cycles, suggest J. W. King, E. Hurst, A. J. Slater, P. A. Smith and B. Tamkin. The forthcoming World Food Conference in Rome might well wish to discuss implications for global agricultural planning.

in any of the previous eight years; in these eight years an average of 83% more wheat was produced than in 1957.

The facts show that the modulation of wheat production associated with the 11-year sunspot cycle seems to be at least 10% in many parts of the world; in certain countries it may even be greater than 50%. A consequence of this modulation may be the fact that the Soviet wheat crop in 1972 was smaller than that of 1971 which was, in turn, less than that of 1970. It is well known that the 'failure' of the 1972 crop led to massive purchases of wheat from the United States and it may reasonably be suggested, in view of the reduced Soviet crops in the years following the 1957-58 solar maximum, that this failure was associated with the decline of solar activity between the 1968 sunspot maximum and the minimum due in about 1974.

Figure 1 shows that the yield per acre of two crops in Britain appeared to be negatively correlated with sun-

spot number during two complete solar cycles between 1937 and 1957. The cycles seem to have been accompanied by a 10% modulation of the yield per acre but it should be stressed that the relationship between agricultural productivity and the sunspot cycle is not always as clear as Fig. 1 would suggest; some reasons for this are discussed later.

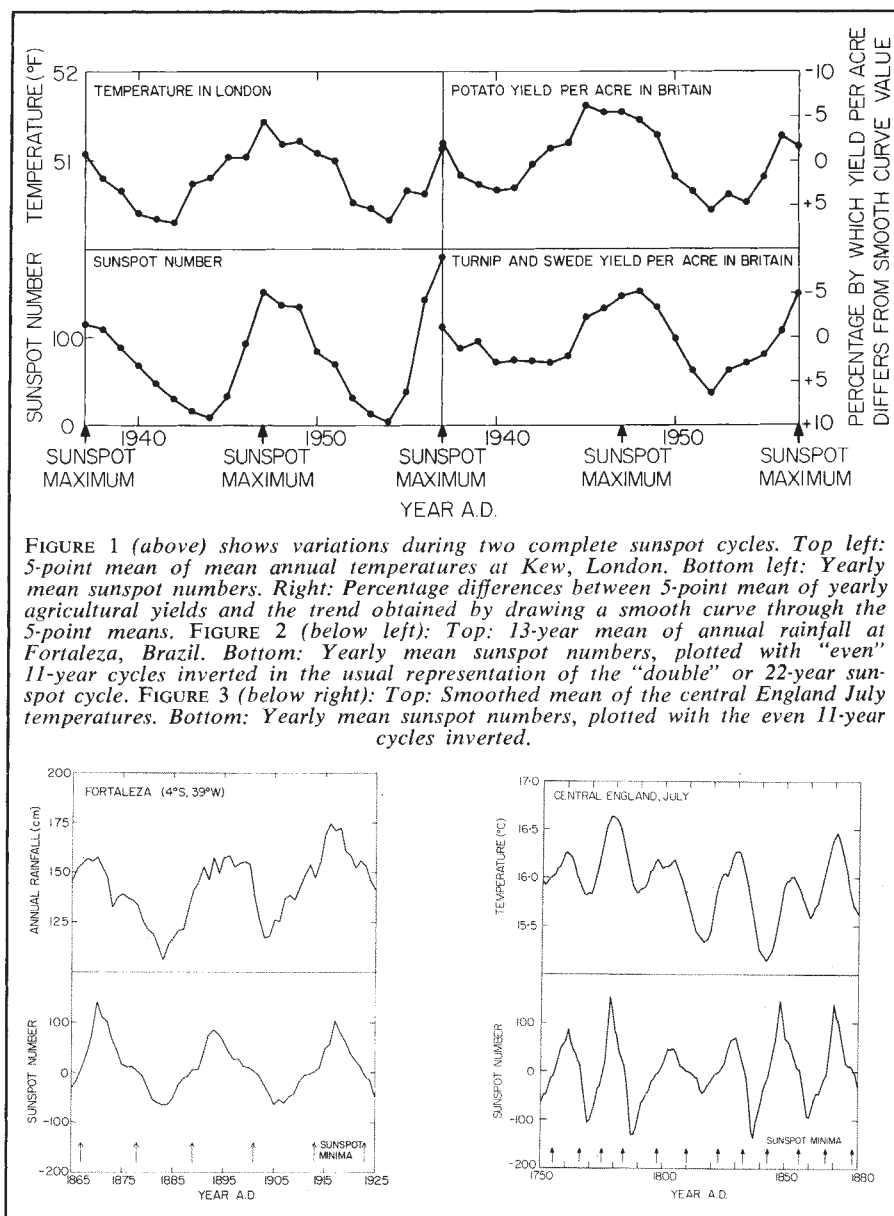
It is not appropriate to describe here the complicated relationships that exist between the solar cycle and the weather in different parts of the world, but some examples of the association between sunspot number, temperature and rainfall are worth mentioning. Figure 2 shows how the rainfall at Fortaleza in Brazil varied during the 'double' or 22-year sunspot cycle; data are plotted for the period 1865-1925 which covered almost three double solar cycles. The rainfall followed the double sunspot cycles closely and the modulation associated with the double cycle amounted to about 35% of the average annual total. P. D. Tyson, as reported in the *Johannesburg Star* in April this year, observed that the rainfall in parts of South Africa exhibits a pronounced modulation (approximately 25% of average annual total) with a period of about 20 years. We have noticed that this oscillation closely resembles the double sunspot cycle and also that the shorter-period oscillation which Tyson observed in the rainfall over the most southerly part of Africa is in antiphase with the 11-year sunspot cycle.

The rainfall in parts of North America is also strongly influenced by the double sunspot cycle. W. O. Roberts¹ has pointed out that droughts in certain regions of the United States that are important for agricultural purposes occur in the years around every second sunspot minimum. The last drought was centred on the sunspot minimum year 1954 and we believe it is significant that the wheat crop in the United States in that year was 12% smaller than the average for the five previous years. It is also significant that in 1974, when the next drought was to be expected, lack of rain has adversely affected the maize crop in the United States. The wheat figures for 1949-73 suggest that Australian wheat production is also influenced by the double sunspot cycle.

Figure 3 shows how the July temperature in central England varied during the 130 years from 1750-1880; evidently a modulation of about 0.75° C occurred in phase with the double sunspot cycle except for a short period around 1850 when the phase of the temperature variation jumped by half an 11-year cycle. More recently, the growing

season (a measure of temperature) in Scotland has undergone a 10% fluctuation in phase with the 11-year sunspot cycle. The rainfall in England also appears, during the past 80 years at least, to have been influenced by the 11-year cycle. Data from Kew (1891–1930) and Bedford (1931–70) show that the rainfall there underwent a modulation in phase with the 11-year cycle. Averages for periods of four years around the sunspot maxima and four years around the sunspot minima (the extreme year of the solar cycle being the third of the four years in every case) show that at Kew the average rainfall at sunspot maximum (four sunspot maxima, 16 years) was 17% greater than at sunspot minimum (three sunspot minima, 12 years). At Bedford the rainfall at sunspot maximum (16 years) was 16% greater than at sunspot minimum (16 years). It does not seem unreasonable to suggest, since the 11-year sunspot cycle appears to modulate both the rainfall and the temperature over Britain, that agricultural productivity may be affected by the solar cycle.

The temperature data shown in Fig. 3 provide evidence of a long-period variation which will be described elsewhere, but it should be pointed out here that the phases of the two long-period temperature variations (78 years and 181 years) observed in parts of the Northern Hemisphere are such that the maximum temperatures associated with these variations occurred in 1934 and 1922 respectively; the period around 1930 was thus relatively warm. It is well known that the period around 1700 was relatively cold; it is referred to as the "Little Ice Age". Temperature data published by Manley² for central England can be used to show that the average length of the growing season during the decade 1691–1700 was about 220 days. Temperatures from Kew for the particularly favourable decade 1931–40 show that the average length of the growing season was approximately 280 days and it is evident (without allowing for the slight temperature difference which exists between central England and Kew) that long-term temperature trends are sufficient to cause the growing season in England to vary by about 60 days, approximately 25%. A return to the conditions which existed around 1700 would obviously result, from an agricultural point of view, in a much less favourable environment than that which has existed during the twentieth century. It is worthy of note that a downward trend (based on farmers' estimates compiled each year for six different crops, and published in *The Times*, September 2 this year) appears to have occurred recently in British agricultural productivity. The average estimate (with 100%



indicating 'full growth') for the years 1964–68 was 94.23%, whereas for the years 1969–74 it was only 91.86%. These figures indicate a decline of 4.6% per decade and, although evidence such as this cannot be used to establish the exact connection between agricultural productivity and long-term climatic trends, it does suggest that the gradual return to colder drier weather which has taken place recently may be having an adverse effect on agricultural output in Britain.

The physical processes through which solar radiation changes associated with the 11-year and 22-year sunspot cycles affect the weather have not yet been identified. At present there is not even a general picture, much less an explanation, of how the climatic effects associated with the solar cycles vary over the Earth. It is already known, however, that the 11-year cycle and the rainfall are positively correlated at some latitudes and negatively correlated at

others; such information could be important for agricultural planning purposes. It is also known that the phase difference between the solar cycle and the rainfall variation at particular locations undergoes sudden jumps of up to 180° but the pattern, if any, of such changes is not known.

Much more work needs to be done to establish the spatial morphology of the various climatic effects associated with the sunspot cycles. It seems reasonable, however, to expect that during the next decade man's knowledge of the effects of the 11-year and 22-year cycles on the weather may advance sufficiently for some of these to be taken into account in global agricultural planning. We thank Mr E. H. Locke and Professor P. D. Tyson for supplying data.

¹ Roberts, W. O., *Proc. Symp. on possible relationships between solar activity and meteorological phenomena* (NASA, 1974).

² Manley, G., *Q. Jl. R. Met. Soc.*, **100**, 389 (1974).

international news

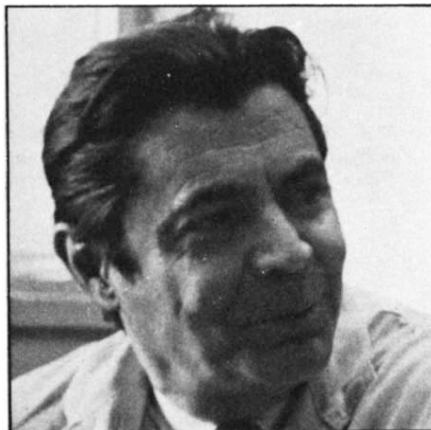
MODERN cell biology can now be considered fully established as a rigorous experimental science with the award of this year's Nobel Prize for Medicine and Physiology to Albert Claude, Christian de Duve and George Palade. No other group of biologists has done more than these three laureates to advance our knowledge of the spatial organisation within the cell of diverse biochemical functions—particularly in higher organisms.

To Albert Claude, now 75, it must be particularly gratifying to see this recognition of his pioneering work begun nearly 35 years ago. It was at the Rockefeller Institute (now Rockefeller University) in 1940 that Claude, having recognised the inadequacy of light microscopy and histochemical techniques then available for correlating intracellular structures with their biochemical functions, began to apply electron microscopy to biological material. Also in the early 1940s, he devised the first successful procedure for resolving cellular organelles by the technique of differential centrifugation.

It was the combination of electron microscopy and differential centrifugation of disrupted cells that rapidly laid a foundation for cell biology as we know it. Claude recognised the importance of treating cells with utmost gentleness, both in preserving their structure and during their disruption, in order to gain further insight into the relationship between morphology and function of individual intracellular components. This was soon rewarded by the electron micrographs, taken with K. Porter and E. Fulham which revealed the ultrastructure of cultured animal cells, as well as by the first useful method for biochemically studying isolated mitochondria. The examination of isolated mitochondria in the electron microscope and the analysis of their characteristic enzyme activities in the test tube validated the biochemical studying of isolated subcellular function *in vivo*, although there were many who remained sceptical of this approach. Claude soon extended the principle of differential centrifugation to derive a particulate fraction from rat liver cells which were less dense than mitochondria and which he called "microsomes". It was another decade, however, before their intracellular origin and their importance in protein synthesis and secretion, as well as in drug detoxication, were fully realised.

Nobel Prize 1974

George Palade



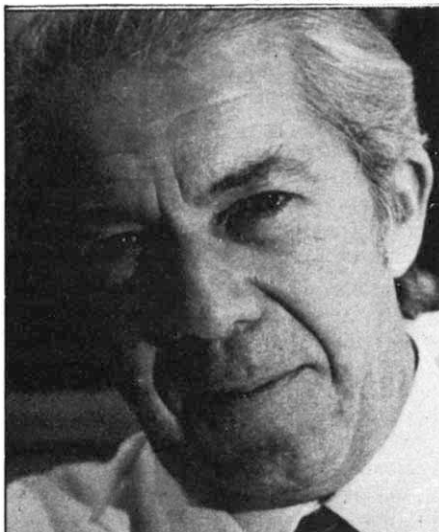
Claude's mitochondria isolated from rat liver were heavily contaminated with other subcellular components and the procedure of differential centrifugation was improved in 1948 by his students at the Rockefeller Institute, Hogeboom, Schneider and Palade. Their procedure forms the basis of all the variants used at present in biochemical laboratories throughout the world for preparing active mitochondria and it is hard to overemphasise the impact this development has had on our present concepts of oxidative phosphorylation and biogenesis of mitochondria. Equally im-

portant is the fact that the Hogeboom-Schneider-Palade technique formed the basis for the isolation in an active form of other subcellular organelles, to be taken up by de Duve and Palade.

In 1949, Claude returned to his native Belgium to take up the directorship of the Institut Jules Bordet, an event of some importance to Christian de Duve who had then taken up the Chair of Physiological Chemistry at Louvain. De Duve's group was then struggling in an ill-equipped laboratory with the question of the effect of insulin on glycolysis in liver tissue homogenised brutally in a kitchen blender, with "typical disregard of cellular organisation", as de Duve himself expressed a few years later. But Claude's presence was immediately felt when the Louvain group (which included H. Hers and J. Berthet) adopted the swing-out ultracentrifuge rotor for investigating the close association of certain groups of hydrolytic enzymes in rat liver. This soon led to the discovery in 1955 of the lysosome, an organelle in which are located all the most important degradative enzymes of the cell, such as phosphatases, nucleases and proteases. De Duve and his collaborators also demonstrated that the lysosomal enzymes existed in a latent state, but could be activated by physical or chemical injury to the cell or to the isolated particle. This observation has led to the publication of several monographs and thousands of papers on the important role of lysosomes as the central element of an "intracellular digestive tract" involved in turnover of cellular components—an indispensable function during cellular differentiation, development of an immune response, carcinogenesis and cell death. Of all the numerous discoveries by these three Nobel laureates, none has more potential value in medicine and pathology than the lysosome. The full realisation of its potential will, however, depend on determining whether altered lysosomal function is a cause or a consequence of the diseased cell.

In the past few years, de Duve, who has occupied a unique dual position at the University of Louvain and at the Rockefeller University, has also been responsible for the discovery of the peroxisome (whose function *in vivo* is as yet hard to assess) and for the application of the zonal ultra-centrifuge rotor to subcellular fractionation. Perhaps his most outstanding contribu-

Christian de Duve



UNTIL about 35 years ago the unusual properties of long-chain macromolecules—such as rubber-like elasticity, high solution viscosity, abnormally low osmotic pressure—seemed so puzzling that chemists attributed very peculiar and mysterious intermolecular forces to such substances. When it was recognised, however, that covalent structures comprising thousands of atoms could be formed, the quantitative physicochemical sciences of macromolecular systems could be developed without recourse to these mysterious forces. Professor Paul J. Flory of Stanford University, this year's recipient of the Nobel Prize for Chemistry, stands foremost among those who have devoted themselves to the development of the physical chemistry of high polymers. His exacting efforts have provided the basic conceptual framework for much of polymer science. It is Flory's distinction to have made original and fundamental contributions to virtually every phase of the subject. He was a pioneer in establishing the basic concept that research in polymer chemistry can be carried out with the same scientific rigour as in other branches of chemistry.

Professor Flory's first contribution to polymer science consisted of theoretical and experimental investigations of the principles of condensation polymerisation. This pioneering effort in the application of statistical methods to a problem in polymer chemistry was exact and definitive and was extended to more complicated systems with multifunctional reactants. The result was the concept of an infinite network polymer and a statement of the con-

ditions for its formation. In studies of free radical polymerisation, Flory was the first to point out, in 1937, the importance of chain transfer processes in controlling molecular weight and to show how such processes lead to branching and crosslinking.

Another area strongly influenced by Flory has been the study of the physical properties of polymers in bulk. These include major contributions to rubber elasticity theory and experiments, together with the basic understanding of the swelling of insoluble polymer networks. Professor Flory pioneered the measurement of the melt viscosity of polymers and its interpretation in terms of molecular weight, molecular weight distribution, degree of branching and temperature. His paper on the statistical thermodynamics of polymer crystallisation represents a major contribution, which clarified an area that had previously been confused. He was able to show how the crystallisation-melting phenomena involving long-chain molecules fits into the classical framework of a first-order phase transition. From this basic concept has come an understanding of crystallisation kinetics, the recognition of the importance of nucleation processes in crystallisation, morphology and the properties of semicrystalline polymers. These ideas were then applied to oriented (fibrous) systems with the development of a quantitative theory of contractility and tension development in the fibrous proteins and applications to natural systems.

Professor Flory has made major contributions to the determination of the quantitative relations between the

macroscopic thermodynamic and hydrodynamic properties of a polymer solution and the average properties and interactions of the dissolved chain. The genesis of this work was the now celebrated "lattice theory", published in 1942, which gives an explanation for the enormous deviations of the thermodynamic properties in polymer solutions from the ideal mixing law. This work was extended to encompass dilute solutions where intra and intermolecular excluded-volume effects were taken into account. When this work was coupled with an analysis of the frictional properties of dilute polymer solution a unified treatment of viscosity, sedimentation velocity and diffusion resulted. The well-known Flory theta temperature, equivalent to the Boyle Point of a real gas, emerged from this work and prescribed the conditions in which a polymer solution behaves ideally. This major development has allowed for the simple and direct determination of molecular weight and of the conformational properties of chain molecules in solution.

This description of Flory's work, the excellence of which continues to this day, shows that it is the cornerstone of every area of polymer science.

The hallmark of his work is a keen analysis of complex problems leading to essentially simple solutions. The close relationship between experiment and theory is notable in his work. Experimental findings have promoted new theoretical investigations while the predictions of theory have been tested in elegant but simply executed experiments.

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tion to cell biology is his strict adherence to precise quantification and establishing 'balance sheets' for enzymes and other cell components derived by subcellular fractionation, a concept unfamiliar to classical cytologists before Claude's pioneering studies. His elegant use of enzyme markers has led not only to the discovery of the lysosome and the peroxisome but to engendering modern "enzyme cytology".

George Palade, a native of Rumania, was also influenced by Claude when he arrived to work at the Rockefeller Institute in the 1940s. Soon after playing his part in developing a better procedure for isolating mitochondria, Palade innovated techniques for fixation of tissues and subcellular fractions for electron microscopy which have now become standard throughout the world. This enabled him to propose a double membrane model for mitochondria, but by the mid-1950s he turned his attention to "microsomes".

With K. Porter he showed that this subcellular fraction was derived from the endoplasmic reticulum, an intricate network of intracellular membranes and ribosomes. In a fruitful and long lasting collaboration with P. Siekevitz, he provided the first direct confirmation that the ribosome was the site of protein synthesis in the cell. They also showed that active ribosomes existed both as free particles and in a form, predominant in protein secreting cells, in which these are attached to membranes. (Such membranes themselves have since been recognised to be the principal site of metabolism and detoxication of drugs, hormones, carcinogens and so on in the liver.) Students of molecular biology often fail to realise that the foundations for the spectacular success with cell-free preparations from *E. coli* in understanding the process of translation of genetic information were laid down by the work on rat-liver ribosomes in the laboratories of Palade and Zamecnik.

In the 1960s Palade's group became more and more interested in secretion of proteins and, in a series of elegantly designed and executed experiments involving the pancreas, established that proteins for export from the cell are exclusively synthesised on ribosomes bound to the membranes of the endoplasmic reticulum and not on free polyribosomes. They then described the vectorial passage of the newly synthesised protein into vesicles of "smooth" (ribosome-free) endoplasmic reticular membranes followed by their entry into the Golgi apparatus which would be moving towards the periphery of the cell. Most recently, Palade has turned his attention to the biogenesis of membranes and has proposed the mosaic pattern of arrangement of membrane components.

Finally this year's Nobel Prize highlights an extraordinary success story of modern bio-medical research—the Rockefeller University.

JAMSHED R. TATA

Slowing energy growth without tears

by Colin Norman, Washington

ENVIRONMENTALISTS, opponents of nuclear power and scientists working on energy-saving technologies got some good news recently. A massive study, funded by a grant of £4 million from the Ford Foundation, concluded that it may be possible to meet energy demand in the United States for the next 25 years without enlisting further help from such troublesome sources of power as nuclear reactors, offshore oil, oil shale and strip-mined coal from the Rocky Mountains.

The key to that sanguine prediction can be summed up in one word: conservation. But a good many people have already indicated that they don't believe it, and the Mobil Oil Corporation has published an advertisement in the *New York Times* to say so.

Called the Energy Policy Project, the study has one over-riding message—that the United States has a wide variety of energy policy options available and need not be locked into a madcap development of all its domestic energy resources in order to become independent of Middle East oil supplies. In fact, the study argues that such a policy, which was adopted by the Nixon Administration and which has not changed much under President Ford's stewardship, will be inordinately expensive and environmentally destructive.

The most urgent task, the study suggests, is for the government to adopt a set of strong controls to reduce energy demand. Compared with the weak homilies dished up by President Ford during his recent economic message to Congress, when he exhorted his fellow countryman to drive less, wear lapel badges bearing the letters WIN (for Whip Inflation Now) and not to waste food, the Energy Policy Project's recommendations for energy conservation look pretty tough. But, for a country which wastes as much energy as the United States, they shouldn't be too difficult to take.

In essence, the study says that the growth in energy consumption, which has been racing away at about 4.5% in the past few years, can be cut to 2% if the government applies a strong enough hand. Furthermore, contrary to the dire predictions of some economists and industrialists, the study argues that it would be feasible to reduce energy growth to zero by the 1990s without plunging the country into economic stagnation. The four steps which the study says should be taken immediately are:

- Energy prices should be allowed to

rise by eliminating subsidies to industry, by imposing pollution taxes on energy producers and by levying extra tariffs on oil imports.

- New cars should meet compulsory fuel economy standards so that the average American car would get 20 miles to the gallon by 1985. At present, the average gas-guzzling behemoth consumes a gallon of petrol every 14 miles, and some of Detroit's creations boast around eight or nine miles per gallon.

- Strong governmental measures should be taken to put a stop to the construction of buildings which have appalling heat losses. This could be done, the study reckons, by raising federally recommended standards and easing credit terms for making energy-saving modifications to existing buildings.

- Federal funding for energy research and development should be channelled more towards energy-saving technologies, and incentive should be given to industry to apply the technology.

Adoption of those policies, coupled with some good propaganda in support of energy conservation (rather than the ludicrous statements of Nixon earlier this year when he pronounced the energy crisis over), would have the desired effect of slashing rates of energy growth in half, the study reckons. And that would mean that energy demands could be met over the next 10 years without any major new commitment to four controversial sources of supply—increased imports of oil, development of oil shale and coal deposits in the Rocky Mountains (where it is difficult to reclaim the land), nuclear power, and offshore oil drilling along the Atlantic and Pacific coasts and in the Gulf of Alaska.

Even so, energy consumption would increase by a total of about 28% by 1985, with the result that new oil supplies would have to come from the North Slope of Alaska and from secondary and tertiary recovery from existing wells, that coal production must be stepped up from deep mines and from strip mines in areas where reclamation is possible, and that electric power plants which are already being constructed—including nuclear reactors—will have to be brought on line.

But after 1985 it is a different story. If energy use continues to grow even at the reduced rate of 2% a year, at least two of those troublesome sources of supply will have to be opened up. But the point is that by adopting energy conservation measures now, the government can buy itself 10 years in which to choose among the various supply options, on the basis of thorough study of the possible environmental consequences. And, if it is decided that none of them is acceptable, the study says that more stringent conservation

measures could be taken to move the country towards zero energy growth by the 1990s.

It should be pointed out, however, that the Energy Policy Project's analysis flies directly in the face of two key assumptions underlying much of industry's—and also the Administration's—approach to energy planning. First, and most important, the study rejects the notion that energy growth and economic growth march hand-in-hand so that cutbacks in energy demand can only be accomplished at the expense of a healthy economy and a high standard of living. And second, the study explicitly states that *laissez faire* market economics are simply not good enough to regulate energy prices—the government must take an active role in the marketplace.

As for the first notion, according to the Energy Policy Project's director S. David Freeman, who was also energy adviser to Presidents Johnson and Nixon, "energy growth and economic growth can be uncoupled". In fact, the study concludes that cutting energy growth to 2% a year is a more attractive economic option than letting consumption rip at its historic levels. For a start, capital requirements for the energy industry will be \$300,000 million less over the next 25 years than they would be if growth is not curbed. The study also suggests that a gradual reduction in energy growth will actually increase employment because higher prices for energy will tend to substitute labour for energy in industrial processes. Furthermore, apart from the energy industry, industrial outputs will be virtually the same in conditions of reduced energy growth between 1985 and 2000 as they would if recent energy consumption trends were maintained.

As far as zero energy growth is concerned, that would require a strong governmental hand on the tiller and some changes in life style, but again the study predicts that the effects on the economy need not be dire. Such devices as a gradually increasing energy sales tax, expansion of urban mass transit systems, upgrading of rail services and greatly stepped-up research and developmental programmes would be required. The key will be gradual transition to a zero energy growth economy, to avoid major disruptions, and the study says it can be done at the expense of only a very small reduction in the growth of the gross national product.

The study prophetically warns, however, that "merely to discuss 'zero energy growth' is to unleash a torrent of indignant advertising paid for by major industrial interests which benefit from growth in energy consumption".

A Mobil advertisement published on the same day as the EPP report tells its

readers that the report recommends substituting bicycles for cars, and that it would "move more than 1,000,000 people a year to new communities where they would work at the jobs planned for them by the government. The advertisement recommends elimination of energy waste (which it neglects to define), urges the development of new energy resources immediately and suggests that some environmental regulations should be relaxed. Without energy growth, Americans would "sacrifice their living standards", the advertisement implies.

Such sentiments can be found within the covers of the report itself, however. Before being published, the report was circulated to members of the Energy Policy Project's Advisory Board—a collection of individuals from industry, academe, environmental organisations and government—and their views appear as an appendix to the study. The nature of their criticisms is fairly well determined by who they represent.

Thus, Michael McClosky, Executive Director of the Sierra Club, takes the study to task for not recommending negative energy growth, and for being too timid in discussing the environmental consequences of high energy growth. Donald C. Burnham, Chairman of Westinghouse Electric Corporation, disagrees with the suggestion that energy growth and economic growth can be uncoupled. The assertions to that end are "totally unsupported by the facts", he says, and the changes proposed "would result in substantial social upheaval, as well as economic stagnation". But the most outspoken criticisms are offered by William P. Tavoulareas, President of Mobil, who says that the report is distorted and offers a recipe for economic disaster.

Be that as it may, will the study have any effect on government policy? The Administration's attitude towards energy conservation, as stated by Mr Ford in his economic message, is that it is clearly a good thing but that at this stage it should be voluntary. Mr Ford said that he hoped consumers and industry would be able to cut back enough to wipe a million barrels of oil a day off the import side of the trade ledger by 1976 and warned that some mandatory controls would have to be applied if voluntary conservation doesn't work. But he specifically rejected a recommendation from his chief energy adviser that a 30% tax should be slapped on petrol, and Congress would be extremely reluctant to take politically sensitive steps to allow energy prices to rise.

The over-riding policy remains as stated often by President Nixon—that domestic energy resources should be developed as rapidly as possible to offset oil imports. □

It was just a year ago that former President Nixon launched Project Independence with a rhetorical flourish and directed the Federal Energy Administration (FEA) to draw up a policy which will make the United States self-sufficient in energy supplies by 1980. Called the Project Independence blueprint, the FEA's plan will be published early in November, but with Nixon's original timetable long ago declared impossible, it represents more a discussion of options than a blueprint for self-sufficiency.

According to conversations with several energy officials and Congressional sources, the study's chief message is that the United States will have to rely on imported oil to meet its energy demands for at least the next decade, and probably for long after that.

Unlike the Ford Foundation study, the FEA blueprint does not suggest that decisions on whether or not to develop controversial domestic energy resources can be put off for a decade, but it does nevertheless put forward some ideas for energy conservation.

First, however, the blueprint suggests that if energy prices remain at present inflated levels, this alone could depress demand and cause the growth in energy consumption in the United States to drop to about 3% a year by 1985. In addition, measures such as mandatory fuel economy standards for new cars, credits for

insulating buildings and incentives for industry to use energy-saving technologies could bring the growth in consumption close to the 2% level recommended in the Ford Foundation study, the FEA reckons.

One reason why the United States is unlikely to stop importing oil in the near future, the study suggests, is that there is only a limited possibility for substituting some of the country's massive deposits of coal for oil. Such a substitution has been vigorously recommended both by former President Nixon and by President Ford, but the FEA analysis points out that only in electricity generation can coal replace oil to a large extent. Consequently, the study predicts that coal consumption will perhaps double by 1985, where as most previous forecasts have suggested that production will be tripled during the next decade, and that oil imports will be needed to meet demand at least until 1985, even with extensive drilling off the Atlantic and Pacific coasts and in the Gulf of Alaska.

The FEA study has some cautionary things to say about strip mining for coal in the arid western regions, suggesting that it will be environmentally destructive and that the coal will in any case contain large quantities of uranium, which could prove to be a health hazard. Similarly, the study pours cold water on the prospects for exploration of oil shale.

Plans for two more Dutch satellites

from Arie de Kool, Rotterdam

EVEN though the first Dutch satellite, ANS, did not achieve its intended orbit, both astronomers and technicians are in good spirits because the instrument nonetheless works well. This is supposed to prove that Philips and Fokker-VFW may now regard themselves as able space companies. Astronomers are busy reprogramming their computers to make full use of the new orbital parameters.

Such success deserves to be continued—reasons the Dutch National Institute for Aerospace Development, which was established to promote industrial activity in the field. So it proposes two further satellites, both bigger than ANS, and too large to be built within the present decade or launched by a Scout rocket.

'Phase A' preparatory studies and preliminary design studies have been completed for the first satellite, another one devoted to astronomy, in fact an advanced infrared telescope.

The money is supposed to come from the Ministry of Economic Affairs and the Science Ministry. "After all, one should not expect that industry will be prepared to pay for a satellite it did not ask for", said one official of the Economy Department.

The second proposal is for an Earth resources satellite, with the principal objective of plant disease recognition. Most of the money for this one would have to come from the Department for Development Aid. "One of the main problems to be overcome is to convince the developing countries that they do have an interest in cooperation with a neutral country like Holland in this field", said the same government official.

A group of scientists has, however, taken issue with these propositions. They feel that other fields of science take priority over space research and they are particularly convinced that remote sensing techniques, if at all useful, can only serve the owners of large monoculture plantations, not the small farmers who should, they say, be considered as the backbone of the developing countries. □



Round Britain

THE Eastern hemisphere, viewed from the Western hemisphere on a clear morning in October. On the eastern side of the meridian, and to the right of the picture, lies the Great Equatorial Building of the Old Royal Observatory, Greenwich, its open roof shrouded with protective wrappings. And the structure which appears to be floating over the trees of Greenwich Park is the tubular steel framework of a new Onion Dome, in the process of being hoisted to its place over the pinnacle of tarpaulins. The original Onion Dome, made of papier maché over an iron framework, was a familiar part of the Greenwich skyline until it was damaged in the wartime blitz and finally taken down twenty years ago.

● THE British Steel Corporation is said to be thinking of selling its waste heat for district heating schemes. With five major installations, three of which are close to major centres of population, British Steel wastes heat equivalent to about 30 million tons of coal each year—worth about £600m. Any return on this energy which would otherwise go up the flue—or rather down the drain—would be welcome, to say the least.

If British Steel do go ahead and market their waste heat they will add fuel to the discussion between the Central Electricity Generating Board (C.E.G.B.) and Professor J. M. Cassels, Professor of Nuclear Physics at Liverpool University.

The C.E.G.B. say that district heating schemes are not economical at

present because the heat released from most generating stations is only about 10 degrees or so above the ambient temperature—and so virtually useless for domestic heating purposes. This heat could be used if it was rejected from the power station at a higher temperature, but the C.E.G.B. points out that this would mean a reduction in generating capacity which they say would be very expensive to make up. Professor Cassels disputes this point. He believes that for the 100 MW of generating capacity lost when supplying 600 MW of useable heat for a district heating scheme, there would be a 200 MW slackening in electricity demand. So the power lost would not have to be made up by expensive new oil fired generating stations—as the C.E.G.B. claim—and even with the experience of pipelines and ancillary equipment this should drop the price of the heat from the Board's estimate of about 240 pence per Gigajoule (p GJ^{-1}) to less than 200 p GJ^{-1} . Although Professor Cassels has not yet completed his detailed studies, it is at this point he says that district heating becomes competitive.

● THERE is something distressing in deliberate destruction by man of a man-made structure. The decision now understood to have been taken at last week's board meeting of the National Research Development Corporation (NRDC) to dismantle the mile of hover-train test track at Earith in the fens near Cambridge therefore places a sad seal on a government incursion into

adventurous engineering. This move was implicit in the closure of Tracked Hovercraft Ltd early in 1973 and was foreshadowed in a recent White Paper. Maintaining the track on which nothing has run for over a year costs NRDC £128,000 a year. Dismantling costs have been quoted at £200,000—but they will only occur once.

It was ironical that the decision to scrap should have come last week. At the very time, barely a mile away at the Institution of Electrical Engineers, a big international gathering to discuss linear electric motors was listening to Professor Eric Laithwaite (principal pioneer and proponent of their application to high speed land transport) expatiate on his 'magnetic river' development. An afternoon was devoted to linear motors for advanced transport.

The move to dismantle the Earith track at this time may seem doubly cussed, because rescue seems closer at hand than at any time since the announcement in Parliament by Michael Heseltine that the government was ceasing to support tracked hovercraft. A London firm of consulting engineers, Brian Colquhoun and Partners asked NRDC recently for an option on the track, pending more long term decisions about salvaging some of the five years of development work that had centred on Earith. Many people consider that this work gave Britain a lead of several years in the field of sophisticated high speed tracked transport. This study continues and the Colquhoun offer still holds good.

news and views

Earthquakes predicted

IN 1910, H. F. Reid, student of the great San Francisco earthquake and originator of the elastic rebound theory, wrote (in *The California Earthquake of April 18, 1906*, Carnegie Institution of Washington, 1910): "As strains always precede the rupture and as the strains are sufficiently great to be easily detected before the rupture occurs, in order to foresee tectonic earthquakes it is merely necessary to devise a method of determining the existence of the strains; and the rupture will in general occur in the neighborhood of the line where the strains are greatest, or along an older fault-line where the rock is weakest". Unfortunately, as Brune (*Eos*, **55**, 820; 1974) has recently pointed out, Reid's optimistic assessment, though based on an earthquake model which is generally consistent with modern ideas, contains a flaw which severely constrains the accuracy of any prediction method. For an earthquake is a 'critical limit phenomenon' whose critical limit is influenced by a variety of factors in unpredictable ways. A partial analogy might be the behaviour of a strained wire made of inhomogeneous material. If the load producing the strain is gradually increased the wire will undoubtedly break, but when and where will only become apparent when it is too late. If the single wire is replaced by a bundle of wires (faults) with diverse properties, even the line along which the first break (earthquake) occurs will be unknown in advance.

This inherent difficulty goes some way towards explaining why, 64 years after Reid's statement, reliable earthquake prediction has not yet been achieved. Certainly the lack of success is not for want of effort. Conscious of their extreme vulnerability, the Japanese began to take a serious interest in earthquakes as early as the late nineteenth century when eminent seismologists such as Milne and Oldham were persuaded to make Japan their natural laboratory. More formally, the Imperial Earthquake Investigation Commission was established to encourage seismic studies in the wake of the 1891 Nobi earthquake (magnitude unknown, 7,270 killed); and the 1923 Tokyo earthquake (magnitude 8.3, 99,330 killed) gave the political impetus necessary for the formation of the Earthquake Research Institute at the Tokyo Imperial University. Not all the work which followed was aimed directly at earthquake prediction, although there is no doubt that prediction was the ultimate aim. But in 1961 prediction became a collective research goal when a new group dedicated to that end produced the classic report, *Prediction of Earthquakes—Progress to Date and Plans for Further Development*. Here the Japanese spelled out their basic philosophy that successful prediction would probably only emerge from long term monitoring of many different aspects of the seismic environment. Specifically, they proposed a 10-year scientific programme involving tide-gauge observations, levelling surveys, repeated triangulation, continuous observation of crustal deformation, micro-earthquake studies and geomagnetic work.

So far this work has not paid off in any significant way, although it has been responsible for one minor success. During the Matsuhiro series of earthquakes which struck central Japan during 1965–1966, a combination of various monitoring data and intelligent guesswork enabled Japanese scientists to give some warning of the particularly violent

activity of April and August 1966. But it is ironic that, in spite of this success and in spite of Japan's long-term commitment to earthquake studies, the current wave of prediction stems from a phenomenon to which they gave comparatively little attention. In 1962, Kondratenko and Nersesov (*Trudy Inst. Fiz., Zemli*, **25**, 130; 1962) discovered that the velocity of crustal P waves increased after moderately large earthquakes in the Tadzikistan region of southern central Asia. They had analysed over 800 travel-time curves from foreshocks and aftershocks of earthquakes with magnitudes 5 or greater and concluded that the average P wave velocity was 5.3 km s^{-1} before the main shocks and 6.3 km s^{-1} afterwards.

For six years this discovery remained unknown outside the Soviet Union until Savarensky (*Tectonophysics*, **6**, 17; 1968) reviewed the data in English at a conference in Switzerland. Rikitake's (*Earth Sci. Rev.*, **4**, 245; 1968) initial response to this revelation was to imply doubt about the validity of the data on the grounds that a P wave velocity change of 15% would correspond to a change in elasticity of 30%. But more results soon followed. Semenov (*Izv. Akad. Nauk. SSSR Phys. Solid Earth*, **4**, 245; 1969), for example, showed that prior to earthquakes in the magnitude range 3–5, the P wave–S wave velocity ratio (V_P/V_S) decreased by about 6% and then increased again to its normal value just before the main shock. The phenomenon was then taken up by scientists in the United States. A few years later, Aggarwal *et al.* (*Nature*, **241**, 101; 1973) were able to report that they had discovered V_P/V_S decreases of up to 13% preceding New York earthquakes in the magnitude range 1–3. Soon after, Whitcomb *et al.* (*Science*, **180**, 632; 1973) reported a precursory V_P/V_S reduction of 10% for the much larger (magnitude 6.4) San Fernando earthquake of 1971, and showed that the reduction was due largely to changes in V_P .

By this time a remarkable change of attitude among United States seismologists was apparent. Before the early 1960s most of them had tended to fight shy of any connection with earthquake prediction because of what Oliver (*Tectonophysics*, **9**, 283; 1970) termed its close association with "seers, mystics, fortune-tellers and the like" and the "great publicity . . . given to earthquake predictions based largely on quackery of some sort". But three parallel developments during the 1960s brought earthquake prediction a "great increase in respectability". First was the growth of plate tectonics which gave a new understanding to the role of earthquakes in the global context and thus led to an upsurge of interest in the phenomenon (although by drawing attention to the number of quite different environments in which earthquakes occur, it also seemed to suggest that the prediction problem was even more intractable than had previously been supposed).

The second development, again not directly concerned with prediction, was rather more violent. In 1966, Evans (*Mountain Geologist*, **3**, 23; 1966) concluded that a mysterious series of earthquakes which had been plaguing the Denver, Colorado area for the previous few years was directly related to the injection of fluid into a disposal well at the Rocky Mountain Arsenal some 20 km away. In other words, these were man-made earthquakes; and Evans's explanation of them revived interest in other sources of artificial earthquakes, particularly the shocks thought to be associated with the filling of reservoirs (Carder, in *Engineering Geology Case Histories Number 8*, Geological Society

of America, 1970). The discovery of man-made earthquakes inevitably raised the question of how they were produced; and this led to a basic understanding of the part played in earthquake mechanisms by fluids. Meanwhile, several workers, beginning with Mogi (*Bull. Earthquake Res. Inst.*, **40**, 125; 1962) and continuing with Brace (*Tectonophysics*, **6**, 75; 1968) and Byerlee (*J. geophys. Res.*, **72**, 3639; 1967) and others, had been investigating the behaviour of rocks under stress in laboratory conditions, and had come to appreciate the importance of fracturing in rocks in earthquake-prone regions.

All three developments finally came together during 1972–73 in physical models proposed by Nur (*Bull. Seismol. Soc. Am.*, **62**, 1217; 1972), Scholz *et al.* (*Science*, **181**, 803; 1973) and Whitcomb *et al.*—models which differ only in

detail. In that of Whitcomb *et al.*, for example, the pores and cracks in the initially unstrained rock are saturated with fluid. As the stress builds up prior to an earthquake the rock increases in volume (dilates), leading to a decrease in pore fluid pressure and ultimately to a situation in which the pore and crack volume exceeds the fluid volume. In this undersaturated state the rock voids partially contain vapour, which reduces the overall bulk modulus, V_P and hence V_P/V_S . The initial effect of the dilatancy is thus to delay the onset of the earthquake (dilatancy strengthening); but fluid from outside the dilatant volume gradually flows in, returning the volume to its saturated state, increasing V_P/V_S and decreasing the fracture strength until the earthquake occurs.

With a promising prediction technique to be developed



Fig. 1 *a*, Santa Caterina by Pier Francesco Fiorentino; *b*, damaged areas indicated by holographic interferometry.

THE detection of incipient damage and deterioration in oil paintings is important for their conservation and restoration. A technique developed by Italian scientists will make this easier in future. S. Amadesi *et al.* (*Applied Optics*, **13**, 2009; 1974) used holographic interferometry to study paintings on wooden panels. Such pictures have several layers of primer underneath the paint, and these are liable to peel away from the wood. In the early stages the detachment is easier to repair but harder to detect.

Amadesi and his colleagues realised that if a painting were warmed, detached regions would disperse heat at a lower rate than the surrounding areas, so their temperature rise and thermal expansion would be greater.

Painting holograms

from John Walker

These changes could then be observed by taking two holograms at five-minute intervals, using a laser. The principle is like superimposing two almost identical photographs: any differences are immediately apparent. The advantage of holograms is that much smaller differences are observable.

In a test run on a laboratory specimen—primed poplar wood—plastic slivers under the primer were easily located. The crucial test was made on the fifteenth century Italian panel painting shown in Fig. 1*a*. The change in room temperature during

the five minutes between the two holograms was sufficient to indicate some detachments, but localisation was poor. So the painting was warmed to 40°C (not hot enough to cause damage). Many more detachments were then observed, and they were better defined. This is shown in Fig. 1*b*. The 'contour lines' superimposed on the image are interference fringes, and the kinks indicate detached regions. In undamaged paintings these distortions of the fringe pattern do not occur.

The method can also be applied to frescoes and paintings on canvas. It will be increasingly useful, not only to locate detachments already present, but to monitor undamaged paintings to make sure they stay that way.

and an attractive explanatory physical model to be tested, the expected rapid increase in the number of prediction studies has occurred: at least 10 new reports have appeared during the past three months alone. Mazzella and Morrison (*Science*, **185**, 857; 1974), for example, have gone some way towards confirming the view put forward by Scholz *et al.* that V_P/V_S changes should not be the only earthquake precursor attributable to the dilatancy effect. They find that in the middle of April 1973 the resistivity in the vicinity of a strike-slip section of the San Andreas fault south of Hollister (California) suddenly decreased by 10%, subsequently rising again by the end of May to 10–15% above its initial level and dropping back again to that level by 15 June. On 22 June, a magnitude 3.9 earthquake occurred in the region at a depth of 9.5 km. Calculations suggest that the observed variations could have been caused by either a small resistivity change over a large volume or a large change over a small volume; for example, an 80% resistivity change in a region 4 km wide and 2–6 km deep would both explain the data and be physically possible. But either way, as resistivity changes are known to reflect changes in rock porosity and the degree of water saturation, the link with dilatation is not difficult to find.

Although this is the first time that precursory resistivity changes have been associated with strike-slip faulting, the phenomenon is not strictly new in that similar changes of up to 20% were observed by Barsukov (*Tectonophysics*, **14**, 273; 1972) prior to thrust-type earthquakes in the Garm area of the Soviet Union. But what is new is the discovery by Bufe *et al.* (*Geophys. Res. Lett.*, **1**, 221, 1974) that microearthquakes occurring within 10 km of the epicentre of the 1972 Stone Canyon shock (magnitude 4.6) had anomalously large (by 25%) mean focal depths for 60 days preceding the event. Bufe and his colleagues note that it is possible that seismicity really did migrate vertically during this period; but on balance they conclude that the effect is caused by a dilatancy biasing of the foci corresponding to a 10–15% decrease in seismic velocity within a local shallow region.

Moreover, as the Stone Canyon earthquake epicentre lies close to the San Andreas fault within a few kilometres of the area studied by Mazzella and Morrison, this is further evidence of precursors being associated with strike-slip faulting. Last year, doubt about the existence of this phenomenon was expressed by McEvilly and Johnson (*Science*, **182**, 588; 1973) who concluded that there were no P wave changes prior to the 1972 Bear Valley (California) earthquake, although Robinson *et al.* (*Science*, **184**, 1281; 1974) were later able to refute this suggestion and Wyss and Holcomb (*Nature*, **245**, 139; 1973) had earlier demonstrated the existence of precursors to the strike-slip Matsu-shiro events. In short, it is now certain that strike-slip faulting is no less susceptible to premonitory indicators than is thrust faulting. This being so, it is clear that the absence of a P wave residual anomaly in central California, now reported by Wyss (*Nature*, **251**, 126; 1974), cannot be attributed to any general lack of precursors to strike-slip tectonism. Instead, therefore, Wyss has interpreted his data to mean that between San Francisco and Parkfield (California) there will be no magnitude 7+ earthquake during the next 7 years and no magnitude 8+ earthquake during the next 25 years. Here is a true prediction, the accuracy of which can only be judged by future events.

But to return to retrospective studies, Wyss and Johnston (*J. geophys. Res.*, **79**, 3283; 1974) report that the 1966 Seddon (magnitude 5.8) and Gisborne (magnitude 6.1) earthquakes of New Zealand were associated with P wave changes which began 360 days and 550 days before, respectively. By contrast, McGarr (*Science*, **185**, 1047; 1974) shows that a magnitude 3.8 tremor occurred in South Africa in March 1973 was preceded by no such changes. So is there a problem here, or is the South African shock a

special case? Wang (*Nature*, **251**, 405; 1974) has an ingenious explanation for contradictions of this sort, based on results obtained last year by Gupta (*J. geophys. Res.*, **78**, 6936; 1973). Gupta showed experimentally that when sound waves are transmitted through rock (limestone) containing oriented dilatant microcracks, there is little variation in velocities or velocity ratios in directions parallel to the cracks but large variations perpendicular to them. And apparently unaware of Gupta's work, Anderson *et al.* (*J. geophys. Res.*, **79**, 4011; 1974) have now shown theoretically that fluid-filled ellipsoidal cracks should produce the same effect. Wang's point is thus simple; assuming (and it seems reasonable to do so) that the dilatant cracks lie in a single direction (probably parallel to the Earth's free surface), whether or not V_P and V_P/V_S changes are detected will depend on the direction in which the observed waves are travelling with respect to the cracks. Unfortunately, McGarr, aware of the Anderson *et al.* article before publication, shows that this cannot be the explanation for the lack of precursors in South Africa. On the other hand, the South African event was not an earthquake but a mining tremor; and so there may after all be good reasons why it can break the rule without destroying the principle.

So where does all this leave earthquake prediction? The answer seems to be, in quite a healthy state. There are few, if any, genuine contradictions; and the fact that all precursors obey the same time law, with precursor time related logarithmically to earthquake magnitude, strengthens the view that they may be attributed to the same dilatancy mechanism. But routine prediction is not yet possible nor even likely to become so in the near future. The only certain prediction is that in the coming months much will be said on the subject.

PETER J. SMITH

Control of cell growth by the cyclic nucleotide seesaw

NORMAL fibroblasts in tissue culture can exist in two growth states, one of rapid proliferation (growing) and one of relative quiescence (resting). Transition from the resting to the growing state can be produced by a number of stimuli including growth factors present in animal serum, by insulin and by proteases. Once growth is initiated a whole range of events takes place including stimulation of transport of molecules into the cell, increases in protein and RNA synthesis and eventually DNA synthesis. This sequence of events was termed the 'pleiotypic programme' by Tomkins and his collaborators (*Nature new Biol.*, **232**, 206; 1971), and a knowledge of how these events are related is fundamental to the understanding of growth control in animal cells.

Since hormones such as insulin and factors such as serum proteins are not thought to enter the cell, Tomkins suggested that they interacted with the cell membrane to modulate the production of chemical mediators that regulate events within the cell. As cyclic AMP has been shown to be the second messenger of many hormones which act at the cell surface, it was only natural to look for changes in the compound in cells stimulated to grow. A substantial drop in the concentration of cyclic AMP was observed within five minutes of the stimulation with serum of the growth of mouse fibroblasts, concomitant with a large increase in the transport of various compounds into the cell: some twenty hours later cell division occurred. If prostaglandin E_1 (which raises intracellular cyclic AMP concentrations) was added together with serum some of the transport changes and cell division were inhibited. This led to the

supposition that the fall in cyclic AMP concentrations was in some way a trigger initiating the 'pleiotypic programme'.

But it soon became apparent that falls in cyclic AMP did not invariably accompany induction of growth. When lymphocytes were treated with the mitogen concanavalin A no change was observed in cyclic AMP concentrations but the concentration of the related nucleotide cyclic GMP rose (Goldberg *et al.*, *Proc. natn. Acad. Sci., U.S.A.*, **69**, 3024; 1972). This observation led Goldberg to propose that growth was regulated by the balance of cyclic AMP and cyclic GMP in the cell, high cyclic AMP: cyclic GMP ratios being associated with the proliferative state. A recent paper by Rudland (*Nature*, **251**, 417; 1974) documents changes in cyclic AMP and cyclic GMP in a number of cell lines. It shows that non-transformed fibroblasts exhibit large differences in the concentrations of cyclic AMP and cyclic GMP and in the cyclic AMP: cyclic GMP ratios for growing and quiescent cells but that no such difference occurs in transformed fibroblasts. This suggests that there may be a fundamental difference in the control of cyclic nucleotide concentrations between normal and transformed cells. In any event low cyclic AMP: cyclic GMP ratios are associated with proliferation, high ratios with quiescence. This concordance of fact with theory is somewhat marred by a recent report that no changes can be detected in cyclic nucleotide concentrations in chick embryo fibroblasts in several different growth states or when the cells are transformed with a number of viruses (Hovi, Keski-Oja, and Vaheri, *Cell*, **2**, 235; 1974). Whether this reflects differences in the mechanism of growth regulation in different cell types or a defect in the hypothesis of growth control by reciprocal changes in cyclic AMP and cyclic GMP concentrations is not clear at the moment.

Correlations of the concentrations of cyclic nucleotides with growth pattern in the cell are unsatisfactory in that they do not prove that the nucleotides actually control growth processes within the cell. Two approaches to this problem have been attempted. One is to add cyclic nucleotides exogenously to attempt to mimic or block growth stimulation by serum, and the other is to isolate simple factors that will specifically alter one of these nucleotides within the cell. In recent series of papers, Rudland and Seifert and collaborators report results of both approaches. They reasoned that if an increase in cyclic GMP concentration was a signal for cell division then by adding cyclic GMP exogenously it might be possible to persuade quiescent

cells to divide. The experiment (*Nature*, **248**, 138; 1974) showed that of a number of nucleotides tested only cyclic GMP and its analogues or cyclic IMP were partially effective in promoting division whereas serum can stimulate all the cells to divide. Experiments where serum stimulation is blocked by raising cyclic AMP concentrations have been more successful (*Nature*, **251**, 417; 1974). Almost the entire effect can be overcome using prostaglandin E_1 , but experiments using high doses of potentially toxic compounds for long periods should be interpreted with care, especially if growth inhibition results.

Using the other approach Rudland took advantage of the purified protein factor isolated by Gospodarowicz from bovine pituitary (*Nature*, **249**, 123; 1974). This factor, termed fibroblast growth factor (FGF), together with hydrocortisone and bovine serum albumin can completely replace serum in its growth promoting activity in certain lines of BALB/c 3T3 cells. What is particularly interesting is that FGF initiates the pleiotypic programme without a significant fall in cyclic AMP concentration (*Nature*, **250**, 791; 1974)—only a large change in cyclic GMP occurs. Furthermore studies on isolated membranes from BALB/c 3T3 cells show that FGF will specifically stimulate guanyl cyclase, leaving adenylyl cyclase unaffected. These data strongly suggest that increases in intracellular cyclic GMP concentrations may alone be sufficient to initiate growth without concomitant decreases in cyclic AMP. But it still remains possible that FGF initiates some general membrane change leading to cell growth (such as promoting nutrient uptake) and that cyclic GMP remains merely a correlate of such a change. Only by further dissection of the system by isolation of other factors which affect cell growth in a defined way can the problem be understood.

ROBERT SHIELDS

Multiple transfer processes in heavy ion reactions

from P. E. Hodgson

EXPERIMENTAL studies of the inelastic scattering of heavy ions have revealed a pronounced oscillatory structure in the angular distributions, and this has been explained as an interference effect between the direct inelastic scattering and one-step transfer inelastic scattering. Such effects are particularly strong when the interacting ions differ only by the transferred nucleon, for example in the reaction

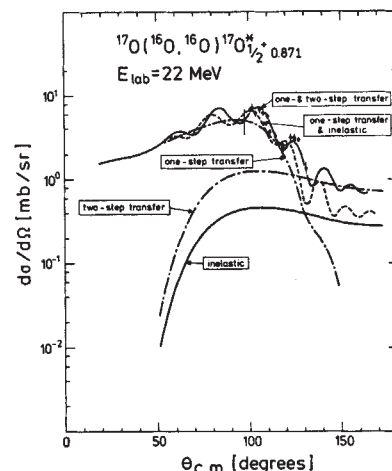


Fig. 1 Differential cross section for the $^{17}\text{O}(^{16}\text{O}, ^{16}\text{O})^{17}\text{O}^*$ ($\frac{1}{2}^+$, 0.871 MeV) reaction at 22 MeV compared with coupled channel calculations of the direct inelastic, one-step transfer and two-step transfer processes, both individually and combined together.

$^{17}\text{O}(^{16}\text{O}, ^{16}\text{O})^{17}\text{O}^*$; so that by the transfer process they effectively exchange their identities (*Nature*, **248**, 735; 1974). Though calculations with this model give a good qualitative account of the data, there remain significant discrepancies, and now Bauer and Wolter (*Phys. Lett.* **51B**, 205; 1974) have shown that these result from the contribution of double transfer processes. Such processes are familiar in the atomic scattering of ions, where electrons are transferred back and forth many times, and some evidence for them has been found in nuclear processes such as the scattering of alpha particles by ^7Li (*Nature*, **246**, 14; 1973).

Bauer and Wolter studied the inelastic scattering reaction $^{17}\text{O}(^{16}\text{O}, ^{16}\text{O})^{17}\text{O}^*$ to the $\frac{1}{2}^+$ state of ^{17}O at 0.871 MeV at several incident energies. They set up a system of coupled wave equations that enabled them to calculate the cross sections of the direct inelastic scattering together with the contributions due to the single and double transfer processes.

The contributions of these processes are compared in Fig. 1 with the experimental cross section for an incident energy of 22 MeV. The direct inelastic process alone gives a smooth angular distribution with its main strength in the backward hemisphere. The one-step transfer alone also gives a smooth distribution but has a much higher cross section and is broadly peaked around 100° . These two processes together interfere to give an oscillatory distribution that agrees qualitatively with the experimental data, but shows a significant phase difference. The two-step transfer alone gives a smooth distribution very similar to the direct inelastic process, but with a higher

overall cross section, and when it is combined with the direct inelastic and the one-step transfer it gives a distribution that agrees very well with the experimental data. The inclusion of the two-step process has thus essentially accounted for the phase difference in the previous comparison without this process.

The full calculation with the one and two step transfer processes as well as the direct inelastic scattering was repeated for some other energies and they were seen to give a very good overall account of the change of the cross section with energy. It is likely that the small discrepancies that remain can be accounted for by higher order transfer processes. The calculations also give the cross sections for elastic scattering, and these agree well with the experimental data.

Flares and spots on dwarf M stars

from R. A. E. Fosbury

PROBABLY the most common self-luminous objects in the Universe are the dwarf M stars: stars which have a mass less than a few tenths that of the Sun but a luminosity which is smaller by a considerably larger factor. Their faintness has, until quite recently, resulted in neglect by observers and theoreticians alike. But this property is an advantage in the study of some curious phenomena which take place in their outer atmospheres. A major flare can increase the brightness of one of these little stars by more than five magnitudes (a factor of one hundred) for a period of a few minutes.

The high kinetic temperatures found in the solar chromosphere and corona are thought to result from the dissipation of a mechanical energy flux which has its origin in the hydrogen convection zone beneath the photosphere. The magneto-acoustic wave modes which are allowed to propagate upwards through the temperature minimum between the photosphere and chromosphere travel through regions of decreasing gas density until they are forced to dissipate their energy as heat through some form of shock. By analogy this form of non-radiative heating is believed to operate in all stars which have a significant convection zone near their surface, that is, those stars which have a spectral type later (cooler) than about F5. Another consequence of the outwardly decreasing density in a stellar atmosphere is the importance of any surface magnetic field in controlling the morphology above the height at which the gas and magnetic pressures are approximately equal. It is the resulting structural in-

homogeneity which makes the quantitative study of the chromospheres of stars other than the Sun so difficult.

The existence of stellar chromospheres can be inferred from observations of spectral lines whose profiles show that the temperature increases outwards through part of their region of formation. The best known of these are the famous violet H and K lines of ionised calcium which can be detected in emission in a very large number of late-type stars. Even more useful chromospheric indicators are the analogous lines of ionised magnesium at 280 nm which are now being observed from balloon, rocket and satellite. A subset of the dwarf M stars, known as dMe stars, show also the Balmer lines of hydrogen in emission and it is from this subset that are drawn most, if not all, of the known flare stars.

The flare stars have now provided the clearest observational evidence so far of the existence of starspots. Observations of quasi-periodic brightness variations (outside times of flare activity) by Evans and Bopp at Texas and others have been interpreted as a rotational modulation caused by large dark areas drifting across the visible hemisphere. These spots can cover as much as 50 degrees in latitude and longitude but the actual sizes they derive depend to some extent on the temperatures assigned to them. One might naively think that the sizes of such surface inhomogeneities are fixed in some way by the pressure scale height in the atmosphere, the ratio of this quantity to the stellar radius being roughly constant along the main sequence. D. J. Mullan (*Astrophys. J.*, **186**, 1059; 1973) however has put forward the hypothesis that sunspots can be considered as convection cells of some type which extend to the bottom of the solar convection zone. In his latest paper (*Astrophys. J.*, **192**, 149; 1974) he extends this idea to the flare stars which, with their position lower down the main sequence, have envelope convection zones with a much greater radial extent. Since the ratio of spot diameter to depth has a minimum value of about two in his theory, he predicts that the diameters of spots should be greater in later spectral types. The predicted sizes are in good agreement with observation.

When the convection zone is confined to a thin shell, as it is in the Sun, the dynamo-generated magnetic field is made up primarily of the higher order multipoles. As the convection zone becomes deeper, however, the lower order multipoles come to dominate until in a fully convective star the field should be mainly dipolar. Mullan suggests that this puts a lower limit on the mass, and hence luminosity, of stars which might be expected to show a rotational

modulation of brightness. If the field is dipolar with rotational and magnetic axes parallel then the spots would be expected to form symmetrically around the poles.

Mullan computes a model for a spot on the flare star YY Gem and estimates a surface field in the spot of about 20 kilogauss. Such large magnetic fields are probably generated by efficient dynamos driven by fast differential rotation. There is some evidence that tidal effects may be partly responsible for triggering the sudden release of energy contained in a magnetic field, resulting in a stellar flare.

Macrophages eat their way into genetic control of immunity

from Robert Kerbel

MACROPHAGES seem to exist solely for the amusement of immunologists, enabling them to re-discover their importance about every five years. This predictable cycle has been upset by recent events, and one could say it's been a good year—or two—for the macrophage.

To begin with, it appears the macrophage has been restored to its position of being the principal aggressor cell in anti-tumour immunity systems (see for example Eccles and Alexander, *Nature*, **250**, 667; 1974) after being temporarily eased off centre stage by the T cell. Then there was the work of Feldmann and colleagues which assigned a role to macrophages in antibody formation beyond the mere digestion and processing of antigen (Feldmann and Nossal, *Transplant. Rev.*, **13**, 3; 1972); these authors reason that the surface of certain types of macrophage is the main site of T and B cell collaboration, by virtue of the highly cytophilic nature of an antigen-specific factor (IgT, as it is called) liberated by activated T cells, for the surface of macrophages.

But at least these are areas in which macrophages have always been thought to play a part, even if the importance of their role varied from one year to the next. A recent set of papers from the laboratory of J. G. Howard, however, now transports macrophages firmly into an area in which they were never seriously considered previously to have a role—genetic control of the immune response (Howard, Courtenay and Desaymard, *Eur. J. Immun.*, **4**, 453; 1974; and Weiner and Bandieri, *Eur. J. Immun.*, **4**, 457; 1974). The authors' model involved the use of so-called Biozzi 'high' and 'low' responder mice; these are two lines of mice (Ab/H and Ab/L) which were genetically selected

from outbred Swiss Albino stock for their respective high and low antibody responses to immunisation with sheep red blood cells (Biozzi *et al.*, *Annls Immun. Inst. Pasteur, Paris*, **115**, 965; 1968). Since the original studies with sheep erythrocytes, a number of reports has shown that this separate responsiveness is maintained when using just about any other antigen, including a few which do not require T cells to give a good response. This has gradually led to the view that the genetic difference in the two strains resides at the level of the B lymphocyte, resulting in a relative inability of Ab/L B cells to differentiate and proliferate to antibody-producing cells following contact with antigen.

The results of Howard *et al.* would seem to put this notion to rest. These authors showed that the antigens levan and dextran B1355, both of which do not require T cells (and both being highly branched polysaccharides), give equivalent antibody responsiveness in the two strains. Furthermore if a haptenic chemical grouping, DNP, was coupled to an antigen which normally gives separate responsiveness (polymerised bacterial flagellin or POL, in this case) then high and low responses to DNP were also obtained, whereas equivalent responses were obtained if the DNP was chemically coupled to levan. This indicates that there is nothing intrinsically defective or different in the ability of B cells with anti-DNP specificity to proliferate and make anti-DNP antibodies in the Ab/L strain. The 'defect' rather is, as the authors say, extrinsic to the lymphocyte population.

What then is the nature of this extrinsic difference? The paper by Weiner and Bandieri suggests that it may reside in the way antigens are handled by macrophages. When peritoneal macrophages were isolated from the two strains and cultured overnight, a striking difference in the morphological appearance of the cells became apparent: the majority of Ab/H macrophages were well spread out, with an irregular cytoplasmic outline and many extensions, whereas most of the Ab/L macrophages assumed a spherical conformation and lacked the many prolongations or extensions so characteristic of macrophages cultured on plastic surfaces. When the authors looked at the way macrophages handled a number of antigens which always give separate responsiveness in the two strains they found these antigens were taken up faster by Ab/L macrophages. Furthermore membrane-bound antigen disappeared rapidly from Ab/L macrophages whereas it persisted in Ab/H macrophages. Intracellular digestion of antigen was more rapid in the Ab/L macrophages and

this may be related to their containing higher levels of lysosomal enzymes.

Thus macrophages from the 'low' responders seemed, if anything, hyperactive. These differences were, however, not observed if the antigen tested with the macrophages was levan. The authors therefore speculate that this hyperactivity might render various antigens more poorly immunogenic in the Ab/L line. Whatever the explanation, it seems that macrophages can express genes which help control antibody responses in these strains.

It also may be of considerable interest to those studying the roles of macrophages in cellular immunity that the Ab/H and Ab/L strains give equivalent responses for virtually all types of such reactions thus far tested, including allograft responses, contact sensitivity to chemicals, and graft-versus-host reactions.

Species richness in geological time

from Peter D. Moore

A CONCEPT which has received widespread support among ecologists is that the species richness of a stable habitat increases steadily through geological time. The justification for such support can largely be traced back to the work of Southwood (*J. Anim. Ecol.*, **30**, 1; 1961) who demonstrated a positive linear correlation between the number of Quaternary subfossil records of various British tree taxa and the number of insect species associated with them. If one assumes that the antiquity and persistence of a tree is reflected by the abundance of its subfossil occurrences, then Southwood's work implies that the longer a tree has been present in these islands in any quantity, the more likely it is that many insect species will be associated with it. The finding suggests that the Quaternary time span has not been sufficient to saturate any tree's resources as an invertebrate microhabitat.

Other data have suggested that the relationship between species richness and time is asymptotic, richness rising rapidly at first, then levelling off. The time scale involved before saturation may be reckoned in decades or centuries rather than millennia. For example, Simberloff and Wilson (*Ecology*, **50**, 278; 1969) exterminated the invertebrate fauna of mangrove islands off the Florida coast and observed that reinvasion occurred in an asymptotic fashion over a relatively short period.

Since Southwood's results are so often used to justify the concept of geological time as a critical factor determining richness, Strong has now attempted to re-analyse Southwood's

data (*Proc. natn. Acad. Sci., U.S.A.*, **71**, 2766; 1974). Strong points out a logical inadequacy in Southwood's basic assumption, in that he confounds the antiquity of tree taxa with their past abundance and distribution. To resolve this confusion, Strong plots each of Southwood's tree taxa on a graph relating the logarithm of taxon range (estimated by the number of kilometre square samples in which it is now found) to the logarithm of the number of associated species (using Southwood's figures). The graph shows a strong positive linear correlation. He also found that the distribution of recently introduced taxa about the regression line is not significantly different from that of the native species. Thus recent introductions are saturated with insect species to the same extent as the natives: otherwise they would have fewer insect species than would be expected from a consideration of their area of occupation.

The conclusion from this work is that the area of distribution of a tree is more important as a determinant of its insect species richness than is its antiquity. Evidently the more abundant a tree species is now the more likely it is to have left many fossils in recent Quaternary sediments; since Southwood did not take into consideration the age of subfossil finds, it is not surprising that he found a positive correlation between fossil abundance and insect species richness.

It is unfortunate that Strong did not use more up to date information concerning the insect species richness of certain trees. For example the estimate for larch of about 15 species is far too low, but most values are probably underestimates and the general form of the relationship would probably not have been altered. Strong is wrong, however, on this basis to deny the assertion by May (in *Stability and Complexity in Model Ecosystems*, Princeton, 1973) that although species richness is well regulated on an ecological time scale the regulated level will increase in geological time. Strong has provided no information on what happens to species richness on a geological time scale except that any change in richness is of little statistical significance in comparison with short-term changes. The variation of species richness over geological periods remains an area for conjecture.

Correction

An error was introduced in the final drafting of the article "Oppression or Altruism in Insect Unions?" (*Nature* **251**, 101; 1974). The (female) workers are of course diploid, like their sister queens, and not haploid as stated.

review article

Development of general relativity

S. Chandrasekhar*

This article is based on an invited talk given at the 1974 annual meeting of the American Physical Society in Chicago on the occasion of the award of the Dannie Heineman Prize for Mathematical Physics, February 5, 1974. (The substance of this lecture has been published for private circulation in the Summer 1974 issue of The University of Chicago Magazine.)

In an invited talk at a meeting of this society which Freeman Dyson gave nine years ago¹, he described the history of the development of the general theory of relativity in the following terms:

"Einstein's theory of gravitation took the world by storm in 1919 for two reasons. In the first place, there was the dramatic prediction of the displacement of star-images by the Sun's gravitational field, the organisation of eclipse expeditions to Brazil and Africa to make the very difficult observations that could test the theory, and the clearly positive outcome of the test. In the second place, there was the appealing philosophical character of the Einstein theory, starting with the postulate of a space-time continuum without any special framework of coordinates to label points of space-time with numbers, so that all the laws of physics should be statements which are true in any possible coordinate system.

To Einstein himself and to many other physicists of the 1920s, including Hermann Weyl and the youthful Pauli, it appeared that this beautiful and successful gravitation theory must be the model for the future development of physics. They expected that further extension of the two principles of Einstein, firstly the representation of physical reality as geometrical in nature, and secondly the insistence on general coordinate invariance, would lead to understanding of electromagnetism and of matter, the chief phenomena that remained outside Einstein's original theory. As is well known, these expectations proved false. The theory of matter and electromagnetism took a totally different direction with the advent of quantum mechanics in 1925. Everybody involved in the development of quantum mechanics forgot rather quickly about gravitation and assumed special coordinate systems without apology. Meanwhile the study of gravitation itself lost interest for physicists because nobody could think of any new observations or experiments. So the theory of gravitation gradually became detached from the rest of physics and was studied only by specialists."

Complementary to Dyson's last statement that the general theory of relativity lost its interest to the physicist because "one could not think of any new observations or experiments" is the statement, which one often hears now, that the renewed interest in general relativity during the 1960s is due to the emergence of new astronomical observations and new planned experiments bearing on the theory. I shall not presume to disagree with these statements. But I must confess that it is not clear to me why some very obvious questions were not asked of general relativity until fairly recently: questions whose

answers could well have contributed to an understanding of the theory itself as distinct from its bearing on possible observations or experiments.

I shall illustrate by some examples the kinds of questions that might have been asked.

Orbit of Mercury

One of the first accomplishments of the general theory of relativity was to show that the trajectory of a test particle in the gravitational field of a central mass is a Kepler ellipse which precesses. The agreement of this predicted precession with that observed for the planet Mercury demonstrated that the theory had successfully met one of Einstein's three crucial tests. The comparison that was made is proper since the approximation of considering Mercury as an infinitesimal test particle in the gravitational field solely of the Sun is an extremely good one. It is well known, however, that in the framework of the Newtonian theory, two finite spherical masses will also describe Kepler ellipses, exactly, about their common centre of mass. On this account, it would be natural to expect that in the framework of general relativity these Kepler ellipses will, in a first non-trivial approximation to the theory, also precess; and, further, that the precession will be given by the same formula that is applicable to the case of an infinitesimal test particle except for the difference that the mass of the central body is replaced by some reduced mass. If these expectations should be justified, then their establishment in the framework of the theory cannot be very difficult. Nevertheless, only twenty years after the founding of the theory was the question asked and its solution attempted by Eddington and Clark, Einstein, Infeld, and Hoffmann, and Fock, independently.

Gravitational radiation

Let me consider a second example. The principal reason why Einstein felt compelled to develop his general theory was, of course, the fact that the Newtonian theory is based on instantaneous action at a distance—a proposition that is contrary to the tenets of special relativity. On the general theory, gravitational forces are propagated with the velocity of light, and as a consequence it would be natural if the theory predicted the emission and propagation of gravitational waves by systems that are nonstationary. Simple considerations of a semi-heuristic nature show, as Einstein in fact showed in 1918, that on a linearised version of the theory, gravitational radiation will be emitted by systems that are nonstationary; that such radiation will have a quadrupole character; and finally that the flux of such radiation will be proportional to the square of the third time derivative of the quadrupole moment of the system. Nevertheless, for some forty years serious theoretical doubts were entertained with regard to the reality of the predicted radiation. For example, in the Born–Einstein letters that have recently been published² there is an undated letter

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from Einstein (there is circumstantial evidence that it was written in 1936) in which he wrote:

"Together with a young collaborator I have arrived at the interesting result that gravitational waves do not exist though they had been assumed a certainty to a first approximation. This shows that the nonlinear general relativistic field equations can tell us more or, rather, limit us more than we have expected."

Again in the last page of Infeld and Plebanski's book on *Motion and Relativity* published in 1961, the statement occurs: "the radiation can be eliminated by a choice of coordinate systems;" and the authors quote Einstein as having said: "We do not have any satisfactory classical theory of radiation. Ritz understood this fact; and he was an intelligent man."

That was in 1961.

To clarify the doubts that were expressed about the reality of the gravitational waves one had only to ask the following question: Do the equations of motion of a system, when carried out systematically to the requisite order, include terms which may be described as expressing radiation-reaction in the precise sense that these terms contribute to a secular decrease of a quantity, which we may call energy, and which is conserved by the system in the immediately lower order approximation to the equations of motion?

A remarkable paper³ published by Andrzej Trautman in 1958 would have resolved this question unambiguously but for an unfortunate oversight. The oversight, when corrected⁴, confirms the expectation. The problem was in fact fully resolved in 1962 by the fundamental investigations of Bondi⁵ and Sachs⁶ from a different point of view which I shall not elaborate. After their work, no doubts—that is, no theoretical doubts—were entertained with regard to the reality of the predicted gravitational radiation by systems with time-dependent quadrupole moments.

Schwarzschild

Finally, I shall consider a third example of how one not only did not ask of general relativity questions which would have clarified its meaning but actually argued against its implications.

The solution of Einstein's equations appropriate to the field outside a central spherical distribution of mass was obtained by Karl Schwarzschild in a paper communicated⁷ to the Berlin Academy by Einstein on January 13, 1916, just two months after Einstein himself had published the basic equations of his theory. The circumstances under which Schwarzschild derived his now famous solution are not generally known. I shall digress to tell the story, particularly as 1973 was the 100th anniversary of Karl Schwarzschild's birth.

During the year and summer of 1915, Karl Schwarzschild was with the German army at the Eastern Front—that was during the First World War. Richard Courant once told me that he had met Karl Schwarzschild proceeding to the Eastern Front while he, as a member of the general staff, was with a party retreating from the same front; and Courant said that he was surprised that someone as distinguished as Karl Schwarzschild would be proceeding towards a front which was considered too dangerous for the general staff. In any event, while at the Eastern Front with a small technical staff, Schwarzschild contracted an infectious disease, and he returned to Germany late in the fall of 1915 very ill. He died on May 11, 1916. As Eddington wrote in a notice published later that year⁸: "Schwarzschild's end is a sad story of long suffering from a terrible illness contracted in the field borne with great courage and patience."

But it is not generally known that it was during the period of his last illness that Schwarzschild wrote two of his greatest papers—papers that are great by any standard. One of them⁹ was devoted to the theory of the Stark effect and the foundations for the interpretation of band spectra on the Bohr-Sommerfeld

theory, and the other was devoted to obtaining the solution of Einstein's equations appropriate to static systems with spherical symmetry.

Now Schwarzschild's solution for the metric external to a spherical source of total inertial mass M , as he gave it, and as it continues to be written, is

$$ds^2 = - \left(1 - \frac{2GM}{c^2 r} \right) c^2 dt^2 + \left(1 - \frac{2GM}{c^2 r} \right)^{-1} dr^2 + r^2 (d\theta^2 + \sin^2\theta d\phi^2)$$

Using this metric, Schwarzschild derived the formulae for the precession of the perihelion of Mercury and for the deflection of light, which Einstein had derived earlier by solving the field equations in an approximation beyond the Newtonian.

It will be observed that Schwarzschild's solution has an apparent singularity at

$$r = R_s = 2GM/c^2$$

This is the Schwarzschild radius. Schwarzschild himself was puzzled by this singularity, and he resolved the puzzle for himself in this way.

Since Schwarzschild had considered the metric as describing the field external to a static spherically-symmetric distribution of mass, he investigated in a second paper¹⁰, communicated for publication a month later (February 24, 1916), the solution of Einstein's equations appropriate to the equilibrium of a static sphere of constant energy density. He showed that the radius of such a configuration can never be less than $1.125 R_s$, and he was satisfied with this result since the singularity of the metric at $r = R_s$ will not be relevant under the circumstances he had contemplated. It was proved by Birkhoff some years later (1923), however, that a spherically symmetric solution of Einstein's vacuum-equation must necessarily be static, and that Schwarzschild's solution will apply even if the source, spherically symmetric, were not static. Schwarzschild's lower limit, $1.125 R_s$, is not applicable under non-static conditions, and we are required to examine more carefully whether the surface has any deeper meaning. I turn to this matter now.

That there is no real singularity of the geometry of space-time at $r = R_s$ was realised very soon. In fact, Eddington showed in 1924 that by writing the Schwarzschild solution in a different coordinate system one can avoid even the appearance of a singularity at $r = R_s$ along future directed time-like trajectories.

The real significance of the surface $r = R_s$ becomes clear when one examines the family of geodesics in the Schwarzschild metric. The discussion is indeed straightforward, but it was explicitly and fully carried out only in 1958 by C. G. Darwin¹¹. In retirement, as he was then, Darwin recalls a paper of his written in 1913 on "Orbits of Electrons" in the field of a nucleus and starts his study with the remark that it might be interesting to examine the analogous problem in the framework of general relativity. Darwin further notes that the analysis could easily have been made forty years earlier. So it might have been, but the problem would have appeared too simple to the 'specialists' that Dyson refers to in the statement I quoted at the outset.

The geodesics in the Schwarzschild metric have been discussed and analysed at great length since Darwin's papers. For the present it is sufficient to draw attention to two particular results. The first is that all photon orbits coming from infinity with an impact parameter less than $3^{3/2} R_s$ must necessarily enter the Schwarzschild sphere and arrive at $r=0$, that is, as one says now, these geodesics are future incomplete. The second result is that trajectories, null or time-like, not only must end at $r=0$, but must do so in a finite proper time as measured by a comoving observer. For these reasons the surface $r=R_s$ is called an *event horizon*.

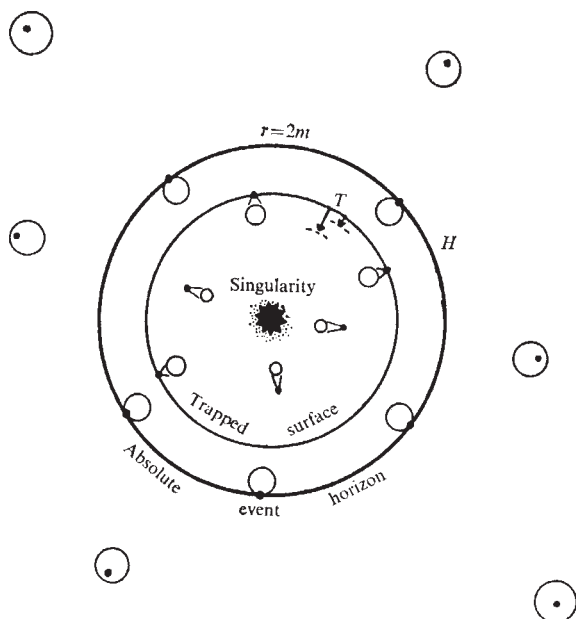


Fig 1 Nature of space-time in the neighbourhood and in the interior of the Schwarzschild radius.

A true picture of the nature of space-time in the neighbourhood and in the interior of the Schwarzschild radius is obtained in the following manner of illustration due to Roger Penrose. In this picture (Fig. 1), the wave fronts of light signals emanating at various points at a slightly later time are shown. At large distances from the event horizon, the points lie at the centres of the respective wave fronts. At smaller distances the wave fronts are displaced by the strong gravitational field present. The wave fronts emanating from points on the event horizon touch the event horizon on the inside and cannot emerge. Inside the event horizon the centre of emission is no longer even inside the wave front; consequently, once inside the Schwarzschild sphere, one cannot communicate with the world outside; and moreover, one would inexorably be propelled towards the centre: not all the King's horses nor all the King's men can prevent it from happening. It should be noted that to deduce all these properties, one has only to draw the light cones at various points—a construction that is entirely elementary.

In essence the significance of the Schwarzschild surface at $r = R_s$ must have been known to Eddington, certainly by the early 1930s; but nevertheless he would not accept a conclusion which appeared inevitable.

That there is an upper limit to the mass of degenerate stellar configuration had been established¹² by early 1931. Its significance for the final stages of the evolution of stars was fully appreciated at that time as the following extract from what was written at that time shows¹³:

"The life history of a star of small mass must be essentially different from the life history of a star of large mass. For a star of small mass the natural white-dwarf stage is an initial step towards complete extinction. A star of large mass cannot pass into the white-dwarf stage and one is left speculating on other possibilities."

And Eddington fully realised what this conclusion implied. He stated¹⁴:

"The star apparently has to go on radiating and radiating and contracting and contracting until, I suppose, it gets down to a few kilometres radius when gravity becomes strong enough to hold the radiation and the star can at last find peace."

This remark makes it clear that Eddington realised that the

existence of an upper limit to white-dwarf configurations inevitably requires that in the course of evolution of at least some of the massive stars, black holes—as we now call them—must form. But he would not accept the conclusion. For he continued to say:

"I felt driven to the conclusion that this was almost a *reductio ad absurdum* of the relativistic degeneracy formula. Various accidents may intervene to save the star, but I want more protection than that. I think that there should be a law of nature to prevent the star from behaving in this absurd way."

Indeed, since he considered the conclusion derived from the theory of Fermi degeneracy of electrons, allowing for the effects of special relativity, as leading to a *reductio ad absurdum*, he modified the relativistic-degeneracy formula so that finite equilibrium states will be possible for all masses.

It is interesting to speculate why Eddington who was one of the earliest and staunchest supporters of general relativity should have found the conclusion that black holes may occur in nature so unacceptable.

Natural home

The three examples I have described at some length all pre-date the 1960s and are in the context of questions that might have been asked of general relativity, questions whose answers would have contributed to our understanding of general relativity. It will be noticed that the questions one might have asked are precisely questions that have been fully answered during the 1960s in the context of relativistic astrophysics. This is not accidental. In a real sense astronomy is the natural home for general relativity. It has to be so, for the general theory of relativity is a theory of gravity; and in what context, except in the context of astronomy, can one hope to observe manifestations of phenomena derived from strong gravitational fields? In other words, the right physical questions one can ask of general relativity are necessarily in the context of astronomical possibilities.

By saying that astronomy is the natural home for the general theory of relativity, I am envisaging a role for theory in astronomy which is not generally accepted. In my judgment, theory has a double role to play in astronomy: the common one of providing interpretations for observed phenomena; and the uncommon one of providing for astronomy the kind of basis which experiments provide for physics. The latter role is largely unrecognised and largely not practised. But one would certainly recognise this role if one would only stop and realise that unless one can be certain of what one might observe in well defined astronomical contexts, that is, under certain well defined conditions, one could never be sure of any inference that one may draw from observations.

Although I do not wish to go so far, there is an element of truth in an aphorism of Eddington's:

"You cannot believe in astronomical observations before they are confirmed by theory."

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- ¹³ Chandrasekhar, S., *Observatory*, 57, 373 (1934).
- ¹⁴ Eddington, A. S., *Observatory*, 58, 37 (1935).

articles

Remnant arcs and marginal basins in the Cainozoic development of the Mediterranean

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Migrations of trench arcs into older marginal basins determined the evolution of the Mediterranean region after the Cretaceous-Eocene closure of the original ocean. Remnant arcs, which now form Corsica, Sardinia and the Balearic Islands separate the Miocene, Pliocene and Recent sea basins. Some remnant arcs such as the Apuseni Mountains separate ensialic basins; others such as the Kabylides, are welded at the fore continental border.

THE present tectonic structure of the Mediterranean area can only be understood if the geological and structural evolution during the past 100 Myr is considered. Although plate tectonic processes can explain neatly the development of the region there are several problems.

First, the evidence that some basins have opened seems to contrast with a general contraction of this region.

Second, there is a divergence between the continuous alignment of post-Miocene structural units and older discontinuous dispersed elements. That can be explained by assuming a very strong horizontal migration driven by recent arcs, which agrees with the greater length and more pronounced curvatures of the more recent fold belts.

Third, studies of the systematic migration of folded and magmatic arcs in the area suggests that the apparent bipolarity of the main structures is a result of temporally distinct processes¹. Coeval symmetric double arc complexes cannot alone be responsible².

Finally, geophysical and geological data³⁻⁶ suggest that the most recognisable basins (the Mediterranean and surrounding components) spread after a phase of general closure of the ocean. That contradicts the simple idea that the Mediterranean Sea is all that remains of an ocean which has been closing since the Mesozoic⁷.

These problems can be studied using models⁸⁻¹³ of the development of folded, magmatic arcs and back-arc zones at consuming plate boundaries in the circum-Pacific area.

Geodynamic model for the Mediterranean region

The peculiar character of the structural framework of the Mediterranean area is evidenced by the orientation and winding of the folded arcs, many of which are often connected with others of opposite polarity (Fig. 1). Because the original Cretaceous-Eocene arcs are of opposite polarities, two main zones can be distinguished: the Betic-Alpine zone and the Dinaric-Hellenic zone. The original alignments of these palaeoarcs have been disrupted and in places the arcs have been shifted considerably by successive phases

of contraction during the Neogene. The accompanying shift of former internal zones seems to correspond broadly to reverse polarities of Neogene arc trench systems, and also to a maximum expansion of the marginal basins behind the arcs. The alignment of oceanic scars marked by mafic and ultramafic masses (Fig. 1) shows the same pattern. In spite of their structural relationships with other units the original location of these ophiolite scars can be identified fairly easily with the two Betic-Alpine and Dinaric-Hellenic belts which were the true oceanic geosuture. It is debatable whether the two principal alignments of the ophiolitic outcrops correspond to one or two oceanic areas¹⁴ (tentatively: northernmost Pyrenean-Valaisian-Vardar alignment and southernmost Betic-Calabrian-Corsica-Ligurian-Piemontais-Serbian-Subpelagonian alignment). The ophiolites which are in any case relicts of a pre-Cretaceous (generally Jurassic) ocean floor indicate a major oceanic closure during the Cretaceous-Eocene (Abbate, E., *et al.*, unpublished).

The evidence that general contraction of the crust continued after the Eocene suggests that most post-Eocene subduction, and consequently the widest arc migrations, must be related to the subduction of the floors of Cretaceous-Eocene marginal basins rather than to the subduction of the original ocean floor. That is how both the western central Mediterranean, and to a lesser degree the Carpathian area, evolved tectonically.

The migration of the Alpine and Hellenic arcs seems, however, to have developed with a fairly constant polarity since the Cretaceous. So, if the present structure is considered, at least four main zones can be distinguished (from east to west: Hellenic arcs; Carpathian arcs; Alpine arcs; and Apennine-Tell-Rif arcs).

The first two of these arc systems originated from former Dinaric-Hellenic alignment and the others originated from the former Betic-Alpine alignment.

Hellenic and Carpathian arcs

Behind the Hellenic trenches there is a series of magmatic arcs which, together with the Aegean Basin constitute a well known, active, marginal basin arc trench system. They are recognisable as well conserved relicts of older folded and magmatic arcs and although they all show the same polarity they become progressively older northwards.

In the Hellenic area, fore arc contraction up until the Eocene occurred by the closure of the original oceanic floor. We think that the Balkans, with an opposite Cretaceous-Eocene polarity, can be considered as the coeval back-arc thrust Belt of the Hellenic arcs¹⁵.

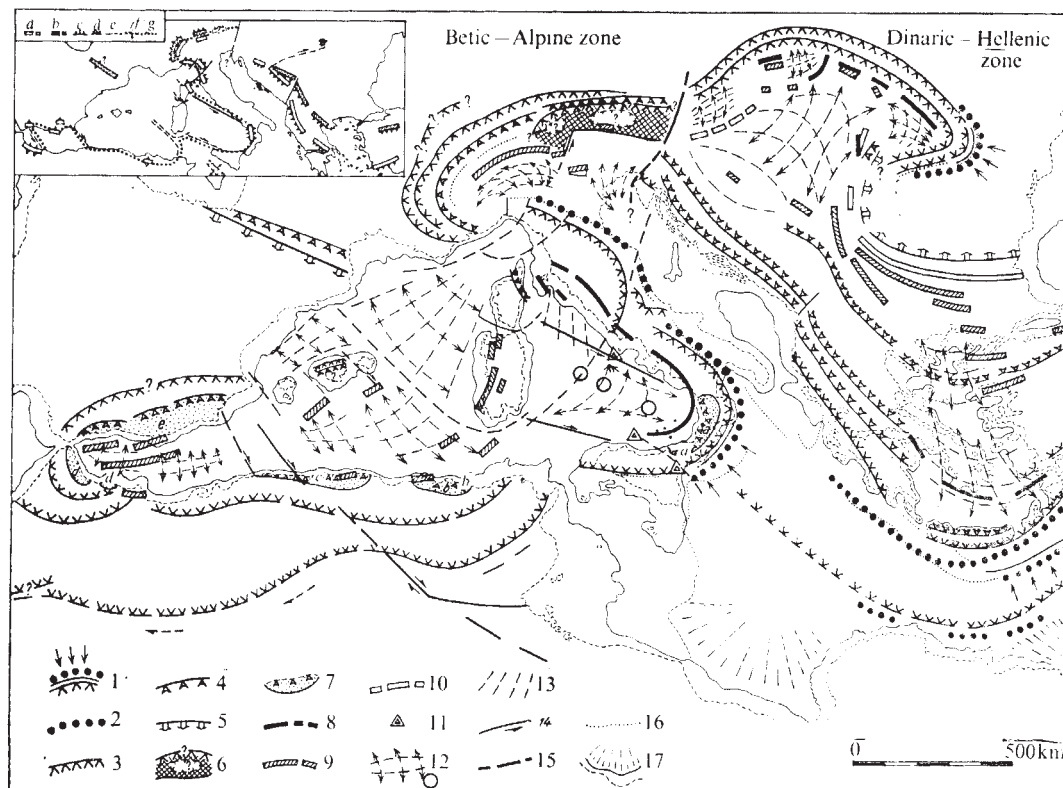


Fig. 1 Geodynamic scheme of the Mediterranean region according to plate tectonic models. 1, Arc-trench systems with active subduction; 2, post-Miocene trench alignments; 3, main Miocene and post-Miocene folded arcs; 4, main Cretaceous-Eocene folded arcs; 5, possible Cretaceous-Eocene back-arc thrust belts; 6, Austro-alpine overthrust; 7, microsilic elements of the same Cretaceous-Eocene Betic-Alpine alignment of the Tellian-Apennine arcs. The original 'mother' continent of these sialic fragments, Austro-alpine⁶ included, could be the palaeo-African plate (see text): *a*, Calabrides; *b*, and *c* Kabyrides; *d*, Sebides and Ghomarides; *e*, Alpujarrides. 8, Post-Tortonian magmatic arcs; 9, Oligo(?)—Miocene magmatic arcs; 10, Cretaceous-Eocene magmatic arcs (relicts); 11, magmatism mainly connected with shear lines; 12, back-arc spread marginal basins with basic magmatism on the floor; 13, pre-spread back-arc distended areas; 14, transform and transcurrent faults; 15, 'Ljubljana-Wien' line separating the Betic-Alpine zone from the Dinaric-Hellenic zone (Cretaceous trench-trench sinistral transform fault and Neogene western limit of the Pannonian and Wien basins); 16, negative gravity anomalies; 17, continental rises and shelves. Inset, correlation and shift of main Mesozoic mafic and ultramafic outcrops: *a*, alignment of Mesozoic ophiolites considered as elements which have migrated from a southern-most oceanic geosuture; *b*, ophiolitic elements which have migrated from a northernmost ocean geosuture; *c*, original polarity of the Cretaceous-Eocene geosutures; *d*, successive polarities during the migration of the reversed arc-trench system; *e*, shear lines; *f*, zone (Colantoni, P., *et al.*, unpublished); *g*, correlation line.

Post-Eocene contraction phases of the arcs developed on the external side, where deep mantle subduction could correspond to the shallow overthrusts of a very thin sialic cover (for instance, the Mediterranean Ridge). Anyway, the type of crust which characterised the floor of the present eastern Mediterranean is still questionable^{4,16}.

Instead, the Carpathian area seems to have developed at the expense of the marginal basin behind the Dinarids, with a general inversion of arc polarity occurring after Dinaric welding during the Eocene. This Neogene contraction in its turn determined the spreading of new first order marginal basins (Pannonian Basin) and second order marginal basins (Wien Basin, Transylvanian Basin, and so on). Active subduction is still evidently associated with the extreme eastern Carpathians¹⁷.

The Apuseni Mountains can also be considered as a remnant arc containing the more internal parts of the inner Dinarids.

Alpine and Apennine-Tell-Rif arcs

The western Mediterranean was formed from Neogene and Quaternary marginal basins^{8,18,19} developed behind the Apennine-Tell-Rif arcs as they migrated southwards and eastwards. The marginal basins, which are of different

stages of maturity are (Fig. 1): the 'Alboran Basin' formed behind the Rif Arc; the first order 'Valencia Basin' formed behind the remnant Balearic Arc; the second order 'Balearic Basin' formed behind the Tell Arc; the first order 'Ligurian Basin' formed behind the remnant Corsica-Sardinia arcs; the second order 'North Tyrrhenian Basin' formed behind the Northern Apennines arc; and the second order 'South Tyrrhenian Basin' formed behind the Calabrian Arc, which is still active.

It is possible to construct a sequence of palinspastic sketch maps to show the possible tectonic evolution of these systems (Fig. 2). There were three phases of Betic-Alpine contraction from the Cretaceous to the Eocene (Fig. 2A, B, and C). The palaeo-European continental margin (of the Atlantic type) includes the sialic fragment of the Balearic Islands in continuity with Iberian sial, and also the Corsica and Sardinia microcontinents (with part of the palaeo-Calabrian sialic substratum) in continuity with the southern French margin. The palaeo-African continental margin (of the Pacific type) is characterised by a festoon of arcs which were migrating northwards together with some detached fragments of African sial. The external ocean floor (Fig. 2A, B) can be correlated with the high pressure and temperature metamorphosed ophiolite suites

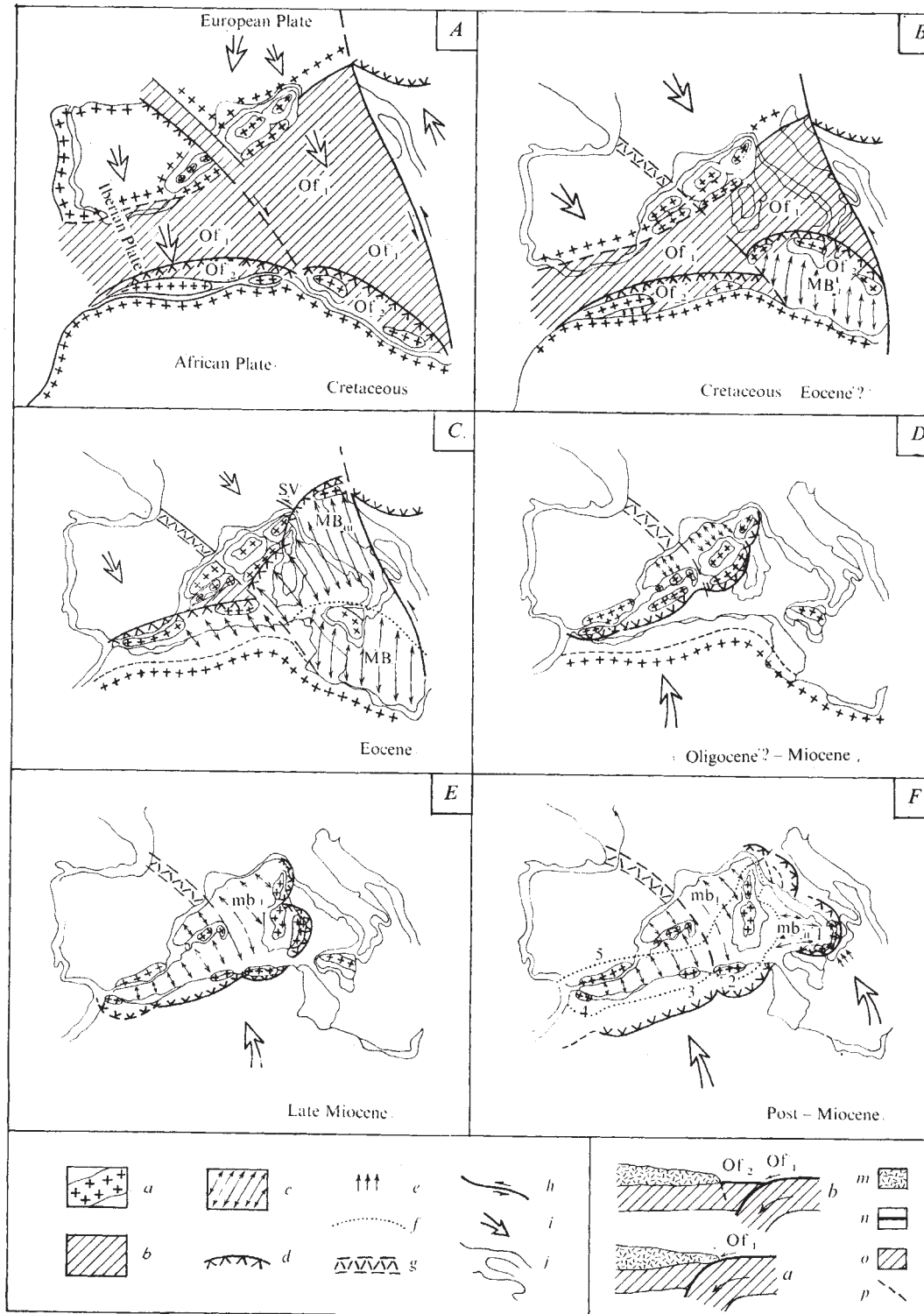


Fig. 2 Hypothetical geodynamic development of the western Mediterranean region. A-C, Cretaceous-Eocene phases of oceanic contraction with the development of northward Betic-Alpine belt up to the arc-continent geosuture, with maximum opening of back-arc marginal basins; D-F, Neogene phases of reversed migration of the southward and eastward Rif-Tell-Apennine arc-trench systems up until the present, with closure of former external marginal basins and opening of new back-arc basins (the actual western Mediterranean basins, see text). a, Continental crust; b, Mesozoic ocean floor; c, marginal basins; d, folded arcs, showing polarity; e, active subduction areas; f, previous position of palaeo arcs for reference; g, Pyrenean geosuture; h, transform faults; i, possible movements of palaeo plates relative to the deep mantle³¹; j, present geographic contours for reference; MB_I and MB_{II}, first and second order Alpine marginal basins; mb_I and mb_{II}, first and second order Apennine marginal basins. 1, Calabrides; 2 and 3, Kabylides; 4, Sebides and Ghomarrides; 5, Alpujarrides. Of-1, High pressure and temperature metamorphic ophiolite suites belonging to the fore-trench oceanic floor (Schistes Lustrés p.p.); Of-2, low pressure and temperature metamorphic ophiolite suites of the back-trench oceanic floor ('Ligurian' units). SV, Sestri-Voltaggio line; m, continental crust; n, oceanic crust; o, upper mantle; p, possible obduction occurrence.

of the *Schistes Lustrés* complex^{20,21}. The internal ocean floor (Fig. 2A, B) can be correlated with low pressure and temperature metamorphic ophiolite suites of the Ligurids complex²².

The arc-continent collision (Fig. 2C) corresponds to the main geosuture phase recorded by the Betic-Alpine belt. The Pyrenean suture (Fig. 2B) has been indicated tentatively at the Cretaceous-Eocene boundary²³.

That was followed by an inversion phase (Fig. 2D) when new arcs, with reverse polarity, became detached and started migrating southwards and eastwards. After the Miocene it seems convenient to speak of a pseudo-Pacific and pseudo-Atlantic type for the European and the African continental margins, respectively, because the original Mesozoic ocean had been practically destroyed during the

Cretaceous-Eocene subduction and had been replaced by a system of marginal basins. The continuing migration of the arc trench systems into older Betic-Alpine marginal basins, caused the opening of new marginal basins in the new back-arc area. These Miocene and post-Miocene marginal basins constitute the present western Mediterranean (Fig. 2E and F).

In this context, Corsica, Sardinia and perhaps the Balearic Islands can be considered as interbasins remnant arcs which more or less had their south-eastern sialic margins 'amputated'. These minor fragments were involved in the subsequent arc migration, and in places contained elements of Betic-Alpine scars and covers (for example, the Calabrian Arc).

Thus, the correlation of Calabrides, Kabyldes, Sebtides, Ghomarides, Alpujarrides and Malaguides^{24,26}, can be carried up to the Austroalpine overthrust (Fig. 1) and they can all be considered as African elements. In summary, they first welded against the European borders during the Eocene (Fig. 2C) and were then either partially bounced back again on to the late Miocene and post-Miocene African border or are still migrating south-eastwards with the Calabrian Arc (Fig. 2F).

Unexplained features

Among the many items requiring explanation is the palaeogeography of branches of the Mesozoic ocean. Was, for instance the Pyrenean oceanic channel continuous with the eventual northern Mesozoic oceanic branch? If so, were the opening and closure of the two oceanic branches contemporaneous? Another point needing clarification is the size and width of oceanic branches which have total estimated dimensions, when assembled, of more than 1,000 km².

The relicts of the Cretaceous–Eocene magmatic arcs are scarcely recognisable in the western Mediterranean region possibly because of the general inversion of subduction directions and the structural counterthrusts in Oligo(?)–Miocene times.

The external zones of the arcs also pose a problem: both the folding of the outermost covers, and the detachment and shear of the foreland carbonatic platforms²⁷ need to be investigated. For the first point we speculate that the well known gliding tectonics could be related, to a certain degree, to the last vertical effect of the previous active subduction. For the second point, a palinspastic reconstruction is necessary. This must take into account synmigrating sedimentation and a progressive decrease and fragmentation of the original carbonate shelves.

Finally, one of the main geological queries concerns the origin of the Mediterranean evaporites, both buried in the present Mediterranean basins and outcropping along many external arcs²⁸.

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New RNA polymerase from *Bacillus subtilis* infected with phage PBS2

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Bacillus subtilis infected with bacteriophage PBS2 contains a new rifampicin-resistant RNA polymerase that is apparently composed of four different polypeptides.

THE growth of phage such as T4 (ref. 1), SP01 (ref. 2) and T7 (ref. 3) requires the DNA-dependent RNA polymerase of the host bacteria. Studies with the drug rifampicin, an inhibitor of bacterial RNA polymerases, have indicated that transcription by coliphage T4 and *Bacillus subtilis* phage SP01 requires the rifampicin-sensitive component of the host RNA polymerase throughout the lytic cycle^{1,2}. On the other hand, the growth of coliphage T7 is only sensitive to rifampicin during the first few minutes of infection³ since this phage elaborates a new drug-

resistant RNA polymerase that directs RNA synthesis late in the lytic cycle⁴. Nevertheless, the host RNA polymerase is initially required for the synthesis of the new T7 enzyme.

PBS2 is a large *B. subtilis* phage whose DNA genome contains uracil in place of thymine⁵. In contrast to the growth of phage T4, SP01 and T7, growth and transcription by PBS2 are resistant to rifampicin throughout the lytic cycle even if the drug is administered to the host bacteria several minutes before infection^{6,7} (T. Fox and S. Clark, unpublished results). A possible explanation for this finding is that when it infects *B. subtilis* PBS2 injects a new drug-resistant RNA polymerase along with the phage genome. Another possibility is that PBS2 utilises a previously undetected rifampicin-resistant RNA polymerase of the host bacteria. To investigate these possibilities we have searched for a rifampicin-resistant RNA polymerase in extracts of PBS2-infected bacteria.

Isolation of enzyme

A new RNA polymerase that copies PBS2 DNA in the presence of rifampicin was first detected in crude extracts of PBS2-infected *B. subtilis* (fraction 1, Table 1). This drug-resistant enzyme was purified partially by ammonium sulphate fractionation (fraction 2) and DEAE-cellulose chromatography (fraction 3). (The enzyme activity at this stage of purification depends completely on added PBS2 DNA.) The enzyme was next separated from *B. subtilis* RNA polymerase by phosphocellulose chromatography. Host and rifampicin-resistant enzymes were eluted by buffers containing 0.4 M and 0.55 M KCl respectively.

Table 1 Summary of purification of PBS2 RNA polymerase

Fraction	$\frac{A_{290}}{A_{280}}$	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Recovery (%)
(1) High speed supernatant	0.53	2890	114	0.04	100
(2) (NH ₄) ₂ SO ₄ precipitate	0.48	2750	162	0.06	142
(3) Pooled DEAE-cellulose enzyme	1.2	800	186	0.23	163
(4) Pooled phosphocellulose enzyme	1.4	6	160	26.7	140
(5) Pooled DEAE-Sephadex enzyme	—	0.48	102	212	89
(6) Pooled glycerol gradient enzyme*	—	0.12	54	450	47

B. subtilis 3610 growing at 32° C in 121A medium⁸ was infected at a multiplicity of three and collected between 40 and 60 min after infection. The enzyme was purified from 60 g of infected cells. PBS2 RNA polymerase activity was assayed in the presence of 20 µg ml⁻¹ rifampicin with 4 µg PBS2 DNA as a template. The reaction mixture was as previously described except that the radioactive nucleotide was ³H-UTP (200 mCi mmol⁻¹) and KCl was omitted⁹. One unit enzyme incorporates 1 nmol of ³H-UMP in 10 min at 37° C. Sonication and high speed centrifugation were as described previously¹⁰. The enzyme was precipitated from the high speed supernatant fluid by addition of ammonium sulphate to 60% saturation and collected by centrifugation at 100,000g for 1 h. The pellet was resuspended in 100 ml buffer C (0.05 M Tris-HCl, pH 8, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% v/v glycerol) containing 0.1 M KCl and dialysed against the same buffer. The dialysed enzyme was diluted twofold and applied to a 500 ml DEAE-cellulose column (5.7 × 20 cm) equilibrated with buffer C containing 0.1 M KCl. The column was washed with 750 ml of the same buffer and the enzyme eluted with buffer C containing 0.24 M KCl. This fraction was applied to a 15 ml phosphocellulose column (1.9 × 5.1 cm) equilibrated with buffer C containing 0.24 M KCl at a flow rate of 30 ml h⁻¹. The column was washed with 50 ml of buffer C containing 0.4 M KCl. PBS2 RNA polymerase was then eluted with buffer C containing 0.55 M KCl. The phosphocellulose purified enzyme was dialysed against buffer C containing 0.01 M MgCl₂ and 0.1 M KCl and further purified by DEAE-Sephadex chromatography and glycerol gradient centrifugation as described in the legends to Figs. 1 and 2.

*The fraction 6 activity and recovery have been normalised assuming that all the fraction 5 enzyme had been applied to the glycerol gradient (see legend to Fig. 2).

The latter eluate (fraction 4) was applied to a DEAE-Sephadex column and eluted with a linear gradient of 0.1–0.5 M KCl (Fig. 1). The rifampicin-resistant activity eluted as a single peak at about 0.33 M KCl (fraction 5). Alternate fractions from the DEAE-Sephadex gradients were analysed by sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis (Fig. 1). Fractions displaying the peak of enzyme activity contained four major polypeptides of 80,000 (I), 76,000 (II), 58,000 (III), and 48,000 (IV) daltons in addition to several other polypeptides in lesser amounts. (Molecular weights were determined by SDS disc gel electrophoresis in phosphate buffer.) As a final purification step, rifampicin-resistant enzyme was subjected to zone centrifugation through a linear gradient of 10–30% (v/v) glycerol. The enzyme (fraction 6) showed a single peak of activity at 11S, coinciding with a peak of protein and indicating high purity (Fig. 2). SDS polyacrylamide slab gel electrophoresis of the glycerol gradient fractions showed that only the four major polypeptides (I, II, III and IV) of the DEAE-Sephadex-purified

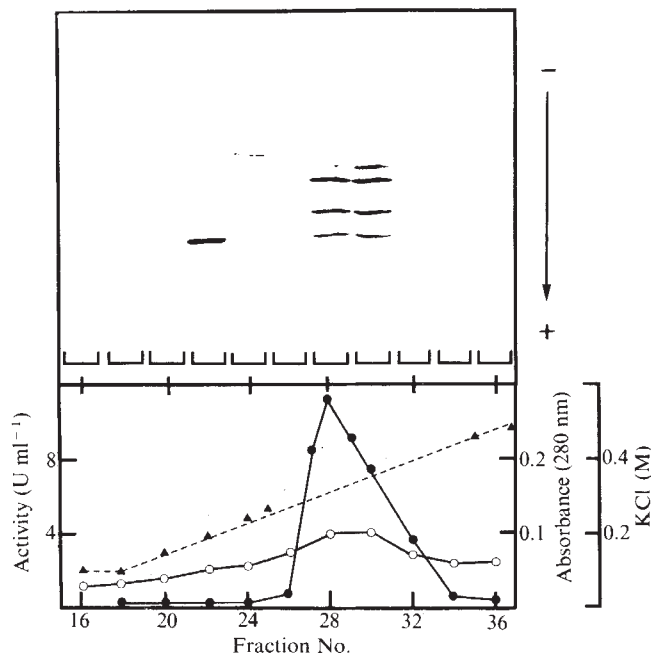


Fig. 1 DEAE-Sephadex column profile. Fraction 4 PBS2 RNA polymerase (6 mg) was applied to a 2.5 ml DEAE-Sephadex column (0.8 × 4.8 cm) equilibrated with buffer C containing 0.1 M KCl and 0.01 M MgCl₂. The column was washed with 20 ml of the same buffer. The enzyme was then eluted with a linear gradient of 0.1 to 0.5 M KCl in buffer C containing 0.01 M MgCl₂ and 5% (v/v) glycerol. Fractions of 2.4 ml were collected and 25 µl was assayed as described in the legend to Table 1 with ³H-UTP (100 mCi mmol⁻¹) (●). Alternate fractions were subjected to SDS polyacrylamide gel electrophoresis (8% w/v acrylamide)¹¹. ○, Absorbance at 280 nm. ▲, Molarity of KCl.

enzyme sedimented coincident with the rifampicin-resistant activity. A densitometer scan of a stained SDS disc gel of the glycerol gradient-purified enzyme indicated that the stoichiometry of polypeptides I, II, III and IV was 1.2, 1.3, 1.0 and 0.9 respectively. None of these polypeptides corresponded to the known subunits (ββ'αα) of *B. subtilis* RNA polymerase (Fig. 3).

Glycerol gradient analysis indicated that the rifampicin-resistant RNA polymerase is a large enzyme that appears to contain four subunits. Moreover, a molecular weight of 262,000 for a tetrameric enzyme containing all four of the polypeptides is consistent with a sedimentation coefficient of 11S. Proof of this subunit structure, however, will require reconstitution of the enzyme from the separated polypeptides. It cannot be excluded, for example, that the rifampicin-resistant RNA polymerase consists of an aggregate of just one of the four polypeptides and the other three polypeptides are contaminants that happen to copurify with the enzyme even after a greater than 10,000-fold purification (Table 1).

In a preliminary experiment rifampicin-resistant RNA polymerase was also purified partially from PBS2-infected *B. subtilis* by a procedure involving partitioning between phases of polyethylene glycol and dextran followed by agarose gel filtration. Two peaks of rifampicin-resistant RNA polymerase activity eluted from the column: one eluted at a position consistent with the size of the glycerol gradient-purified enzyme, and the other was much smaller and could indicate that other rifampicin-resistant RNA polymerases are associated with PBS2 infection but not detected by the purification procedure of Table 1. Alternatively, the rifampicin-resistant RNA polymerase may dissociate under certain conditions, to yield a subassembly that is catalytically active.

Comparison of polymerases

Like the RNA polymerase of *B. subtilis*, the rifampicin-resistant enzyme from PBS2-infected bacteria required all four

ribonucleoside triphosphates, Mg^{2+} and a DNA template for optimal activity (Table 2). The rifampicin-resistant RNA polymerase actively copied PBS2 DNA and the synthetic template poly(dA-dT), but was relatively inactive with other DNA templates (Table 2). The ability to copy poly(dA-dT) indicated that the template specificity of the PBS2 RNA polymerase is not limited to uracil-containing DNAs. To investigate the template specificity of this enzyme further, PBS2 and *B. subtilis* RNA polymerases were assayed with PBS2 DNA and phage Φ e DNA at various concentrations of enzyme and template. The rifampicin-resistant enzyme was more than ten times as active with PBS2 DNA than with Φ e DNA, while the *B. subtilis* RNA polymerase was more than ten times as active with Φ e DNA than with PBS2 DNA (Fig. 4). As an indication of the fidelity of transcription by the phage polymerase, RNA copied *in vitro* from PBS2 DNA by the phage enzyme hybridised with high efficiency (45%) to denatured PBS2 DNA but failed to anneal to denatured Φ e DNA (data not shown).

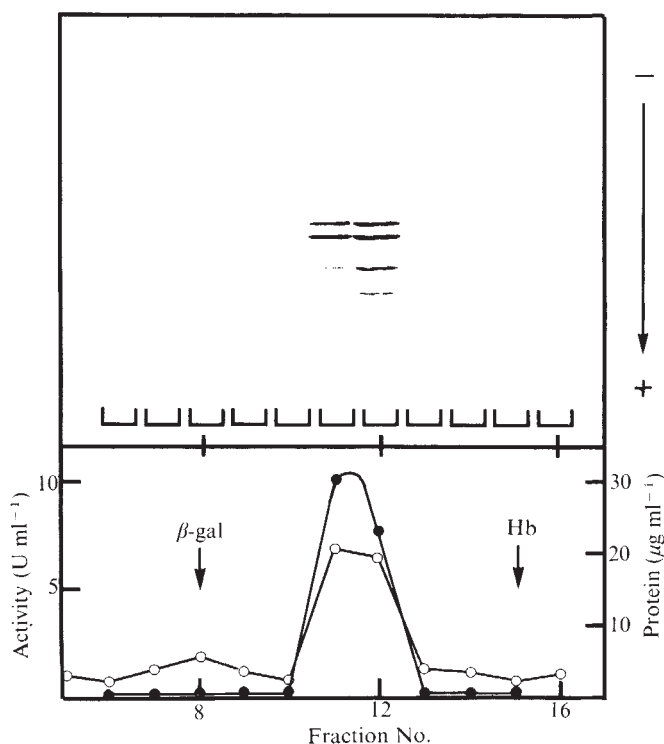


Fig. 2 Zone centrifugation of PBS2 RNA polymerase. Fractions 27 to 31 of the DEAE-Sephadex gradient (12 ml) were pooled and concentrated against Ficoll to a final volume of 2 ml. A sample of 0.5 ml of the concentrated fraction 5 enzyme (0.12 mg) was layered on to a 12 ml linear gradient of 10–30% (v/v) glycerol in buffer C containing 0.1 M KCl and 0.01 M $MgCl_2$ and centrifuged at 2°C for 18 h at 39,000 r.p.m. in an International SB 283 rotor. Sedimentation standards, β galactosidase and haemoglobin, were sedimented in parallel in a duplicate gradient. Fractions of 0.75 ml were collected and 75 μ l was assayed as described in the legend to Table 1 with 3H -UTP (100 mCi mmol $^{-1}$) (●). Each fraction was subjected to SDS polyacrylamide slab gel electrophoresis (8% w/v acrylamide)¹¹. ○, Protein concentration.

The PBS2 and *B. subtilis* RNA polymerases differ not only in template specificities but also in their sensitivities to rifampicin, streptolydigin and antiserum against *B. subtilis* RNA polymerase. The PBS2 enzyme was completely resistant to the drugs and largely resistant to the antiserum, while the *B. subtilis* enzyme was strongly inhibited by each agent (Table 3).

To determine whether the rifampicin-resistant RNA polymerase is present only after PBS2 infection, we tested for its presence in uninfected bacteria, in PBS2-infected bacteria and in bacteria infected with PBS2 after addition of rifampicin.

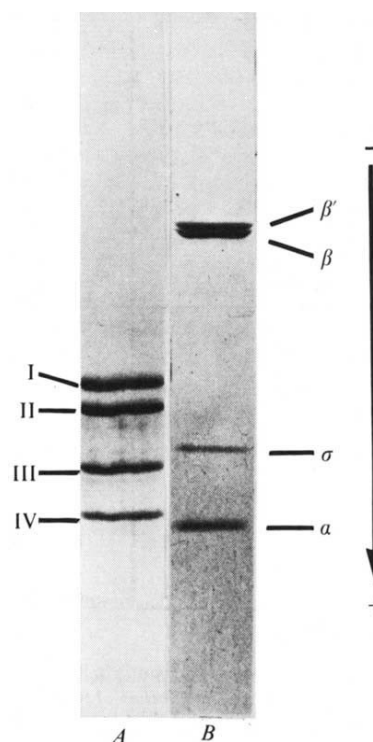


Fig. 3 SDS polyacrylamide slab gel electrophoresis of *B. subtilis* RNA polymerase and PBS2 RNA polymerase. PBS2 RNA polymerase (A) (3 μ g of fraction 6 enzyme) and *B. subtilis* holoenzyme (B) (a mixture of 4 μ g of fraction 5 holoenzyme and 0.5 μ g of fraction 7 sigma, prepared as described previously¹⁰) were subjected to SDS polyacrylamide slab gel electrophoresis (8% w/v acrylamide)¹¹.

Rifampicin-resistant RNA polymerase activity was assayed at the DEAE-cellulose and phosphocellulose chromatography steps of the procedure of Table 1. Only the infected bacteria contained measurable rifampicin-resistant enzyme activity (Table 4). Thus, the PBS2 RNA polymerase is apparently not present in uninfected *B. subtilis* but appears after PBS2 infection even in the presence of high concentrations of rifampicin.

Implications

The isolation of a new rifampicin-resistant RNA polymerase from PBS2-infected *B. subtilis* provides a likely explanation for

Table 2 Requirements for PBS2 RNA polymerase activity

	Activity (%)
Complete system	100
–ATP	<1
–CTP	1
–GTP	9
– Mg^{2+}	6
– $Mg^{2+} + Mn^{2+}$	22
+0.15 M KCl	13
–PBS2 DNA	<1
+poly(dA-dT)	70
+ Φ e DNA	9
+ Φ 29 DNA	17
+ β 22 DNA	4
+ Φ 105 DNA	6
+ <i>B. subtilis</i> DNA	16

Assays were carried out as described in the legend for Table 1. The reaction mixture contained 0.9 μ g fraction 5 PBS2 RNA polymerase. Except where noted, each assay contained 4 μ g PBS2 DNA or 6 μ g of the other templates. For the complete system, 100% activity was 17,000 c.p.m.

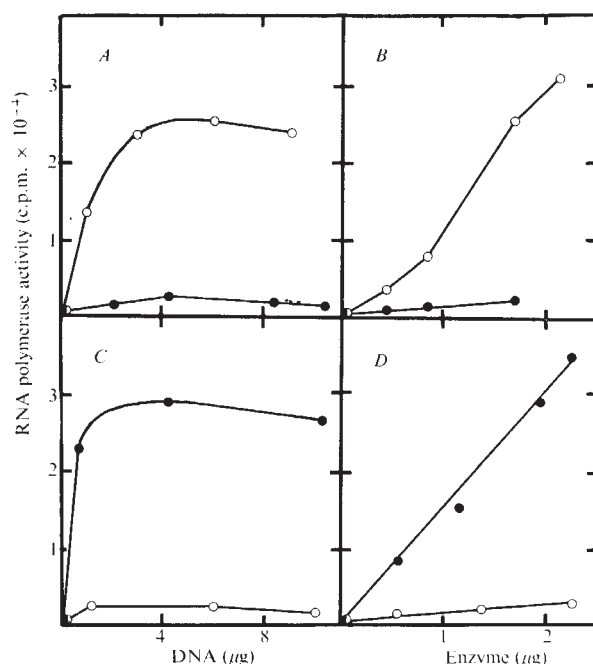


Fig. 4 Template specificity of PBS2 RNA polymerase and *B. subtilis* RNA polymerase. *B. subtilis* holoenzyme (1.7 μ g purified as described in the legend to Table 3) (A) and fraction 5 PBS2 RNA polymerase (1.9 μ g) (C) were assayed as described in the legend to Table 3 with varying amounts of Φ e DNA (○) and PBS2 DNA (●). Various amounts of *B. subtilis* holoenzyme (B) and PBS2 RNA polymerase (D) were assayed with Φ e DNA (6 μ g per assay) (○) and PBS2 DNA (4 μ g per assay) (●).

the drug resistance of phage growth during the later stages of the PBS2 lytic cycle. How is this new RNA polymerase initially produced in cells that have been inhibited with rifampicin before infection? One possibility is that either the PBS2 RNA polymerase, a catalytically active subassembly of that enzyme, or another phage RNA polymerase is encapsulated in the PBS2 virion and injected along with the phage genome at infection. The injected RNA polymerase could then direct the synthesis of additional PBS2 RNA polymerase and other phage proteins independently of the host RNA polymerase. Other possibilities are that the phage injects a protein that confers drug resistance on the host RNA polymerase or that the phage genome is initially transcribed by an unidentified rifampicin-resistant

RNA polymerase of the host. A recent report indicated that transcription by the phage N4 immediately after infection of *E. coli* is also resistant to rifampicin¹³. It will be interesting to compare the mechanisms of drug resistant transcription by these unrelated phage.

The purified PBS2 RNA polymerase is complex in structure and, like the RNA polymerase of *E. coli* and *B. subtilis*, is apparently composed of multiple subunits. Bacterial RNA polymerases are known to interact with phage-induced polypeptides including the protein products of known regulatory genes¹⁴⁻¹⁸. The PBS2 genome is unusually large (1.9×10^8 daltons)¹⁹ and is undoubtedly transcribed in a defined temporal sequence although the programme of phage transcription has not been investigated. It is tempting to speculate that the PBS2 RNA polymerase, like the multi-subunit RNA polymerases of bacteria, interacts with other phage proteins that regulate the expression of different classes of phage genes during the PBS2 lytic cycle.

Table 4 Appearance of rifampicin-resistant RNA polymerase after infection by PBS2

Extracted from	DEAE-cellulose enzyme activity (U mg ⁻¹)	Phosphocellulose enzyme activity (U mg ⁻¹)
Uninfected <i>B. subtilis</i>	<0.01	<0.15
PBS2 infected <i>B. subtilis</i>	0.33	22.7
<i>B. subtilis</i> infected by PBS2 in the presence of rifampicin	0.27	18.3

Three cultures of *B. subtilis* 3610 were grown in medium 121A at 32° C to a density of 2.5×10^8 cells per ml. One culture (25 l) was infected by PBS2 at a multiplicity of three. A second culture (12 l) was infected similarly by PBS2 5 min after addition of rifampicin (50 μ g ml⁻¹). Both cultures were collected 45 min after infection. A third uninfected culture (12 l) was collected at a cell density of 3.5×10^8 per ml. The purification procedure of Table 1 was followed until the phosphocellulose chromatography step. PBS2 RNA polymerase activity was measured as in the legend to Table 1.

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Table 3 Resistance of PBS2 RNA polymerase to inhibitors of *B. subtilis* RNA polymerase

Enzyme	Addition	Activity (%)
<i>B. subtilis</i> RNA polymerase	—	100
	Rifampicin	<1
	Streptolydigin	2
	Antiserum	8
PBS2 RNA polymerase	—	100
	Rifampicin	100
	Streptolydigin	103
	Antiserum	68

B. subtilis RNA polymerase holoenzyme was purified by DEAE-Sephadex chromatography as described previously¹⁰ but omitting DNA cellulose chromatography. *B. subtilis* RNA polymerase (3.4 μ g per assay) and fraction 5 PBS2 RNA polymerase (1.9 μ g per assay) were assayed as described in the legend to Table 1 with ³H-UTP (100 mCi mmol⁻¹ in the presence of 5 μ g rifampicin, 30 μ g streptolydigin or 200 μ g anti-*B. subtilis* RNA polymerase antiserum¹². The template for *B. subtilis* RNA polymerase was Φ e DNA (6 μ g) and the template for PBS2 RNA polymerase was PBS2 DNA (4 μ g). One hundred per cent activity for *B. subtilis* RNA polymerase and PBS2 RNA polymerase was 58,500 c.p.m. and 35,300 c.p.m. respectively.

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letters to nature

Enhanced low-energy gamma rays at balloon altitude in the Brazilian magnetic anomaly

THE region of the Brazilian geomagnetic anomaly presents an interesting opportunity for the study of precipitation of charged particles of the inner radiation belt into the atmosphere below. Some recent studies based on direct and indirect observations (refs 1–3 and our unpublished work) show that the precipitating flux increases appreciably during the period of a geomagnetic storm. The present note provides another evidence in this direction.

As a part of the continuing cosmic ray programme at INPE, balloons with γ -ray and charged particle detectors were launched on October 7 and 20, 1973. They reached the ceiling altitude of ≈ 3.5 mbar and remained there for few hours measuring γ -ray and charged particle fluxes.

The magnetograms obtained at São José dos Campos indicate that on October 20 the balloon was launched during a magnetically disturbed period. It seems that a sudden commencement of small amplitude at about 0645 UT on October 16, 1973, preceded the start of a disturbance in the Earth's magnetic field. It seems that more than one disturbance occurred, so that the period of the balloon flight is a mixture of the main phase and the recovery phase of a magnetic storm. This disturbance continued practically for the whole week. The purpose of this note is to discuss the change in the γ -ray and charged particle fluxes during the disturbed period in relation to those observed during the quiet period in conjunction with relevant geomagnetic and ionosphere observations.

The balloon launchings on both occasions were conducted at São José dos Campos (23°14'S, 45°51'W). On October 7, 1973, the balloon was launched at 0830 UT and it reached a ceiling height of 3.8 mbar but data were obtained for about 30 min only. Even with this short period of observation it is possible to get an idea of the 'normal' level of the γ -ray and charged particle fluxes at the ceiling height. On October 20, the balloon was launched at 0715 UT, attained height of 3.5 mbar at about 0915 UT and telemetered the data obtained for several

hours. Since the ceiling height of both flights is approximately the same it is meaningful to compare the results and look for effects due to the geomagnetic disturbance.

The detector in both flights consisted of a 4"×4" NaI (Tl) scintillator surrounded by a plastic scintillator 1 cm thick. The latter operated in anticoincidence for separating out charged particles. The energy threshold of the detector was such that the minimum energy at the top of the atmosphere of charged particles capable of triggering the detector was 7.0 MeV for electron and 30 MeV for protons. The equivalent area of the NaI (Tl) crystal detector is 121.62 cm². The details regarding the calibration and other relevant information can be found in ref. 4.

The curves of the integral count rates as function of time for γ rays and charged particles measured on October 20, 1973 are shown in Fig. 1. The γ -ray count rate is integrated from 0.9 to 18 MeV. A curve for the pressure measurements is also included in Fig. 1. For comparison the curves for γ -ray and charged particle fluxes in the October 7 flight are also shown in Fig. 1, time shifted so as to superimpose the two curves during ascent, without any change in the vertical scale. Starting from the level of maximum count rate at 120 mbar there is a substantial increase in the γ -ray flux. The charged particle flux also shows noticeable enhancement although not as large as in the case of γ rays. At the ceiling height the charged particle seems to have reached almost the normal equilibrium level.

The increased count rate on October 20 can be attributed to the high level of geomagnetic activity prevailing at that time. (The omnidirectional character of the detector and the short time interval between flights exclude the possibility of interpreting the higher γ -ray count rate on October 20 as being due to contribution from any known or unknown celestial source.)

The fact that the increase in γ -ray flux is considerably larger than the charged particle flux may be attributed to the fact, that the γ -ray flux observed is primarily due to bremsstrahlung produced by the downward moving electron flux. Whereas the charged particle detector will register only the flux reaching the ceiling level, the γ ray counts are due to the integrated radiation produced in the layers above the ceiling height. Since the charged particle flux undergoes a decrease with depth, it would be reasonable to expect that the increase in the low energy γ -ray flux should be larger than the directly measured charged particle flux.

To verify this we have calculated from the measured energy loss spectrum in the crystal, the energy spectrum of the incident γ -ray flux (Fig. 2a). For the period 1100–1230 UT (Fig. 2a) the γ -ray count rate was at a lower level, where it stayed for the remainder of the flight. We therefore assume that this period is representative of normal conditions, when no enhanced particle precipitation is taking place. By contrast, the interval 0900–1030 UT (Fig. 2b) covers the period during which the maximum enhanced precipitation is seen to occur. The spectrum for the period 1100–1230 UT is a power law $\approx E^{-1.1}$ for the entire energy range. For the period 0900–1030 UT the power law is $\approx E^{-2.2}$ in the range 0.9–2.0 MeV and $\approx E^{-1.1}$ in the range 7.0–18.0 MeV. In the range 2.0–7.0 MeV, the spectrum is not well represented by a power law in energy. Thus, there seems to be a relatively greater increase in the low energy flux than in the high energy region. Assuming that the increase in the low energy γ -ray flux is due to bremsstrahlung from electrons with an energy spectrum given by Pfitzer *et al.*⁵ for

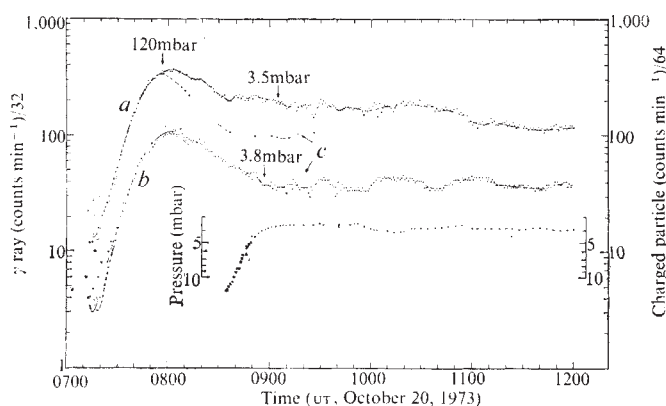


Fig. 1 Count rate as function of time for γ rays (a) and charged particles (b) for the October 20, 1973 flight. Also shown for comparison are the corresponding quantities for the October 7, 1973 flight (c), time shifted so as to coincide with the October 20 curves during the flight ascent. The curve displaying the barometric pressure variation during the October 20, flight is also indicated.

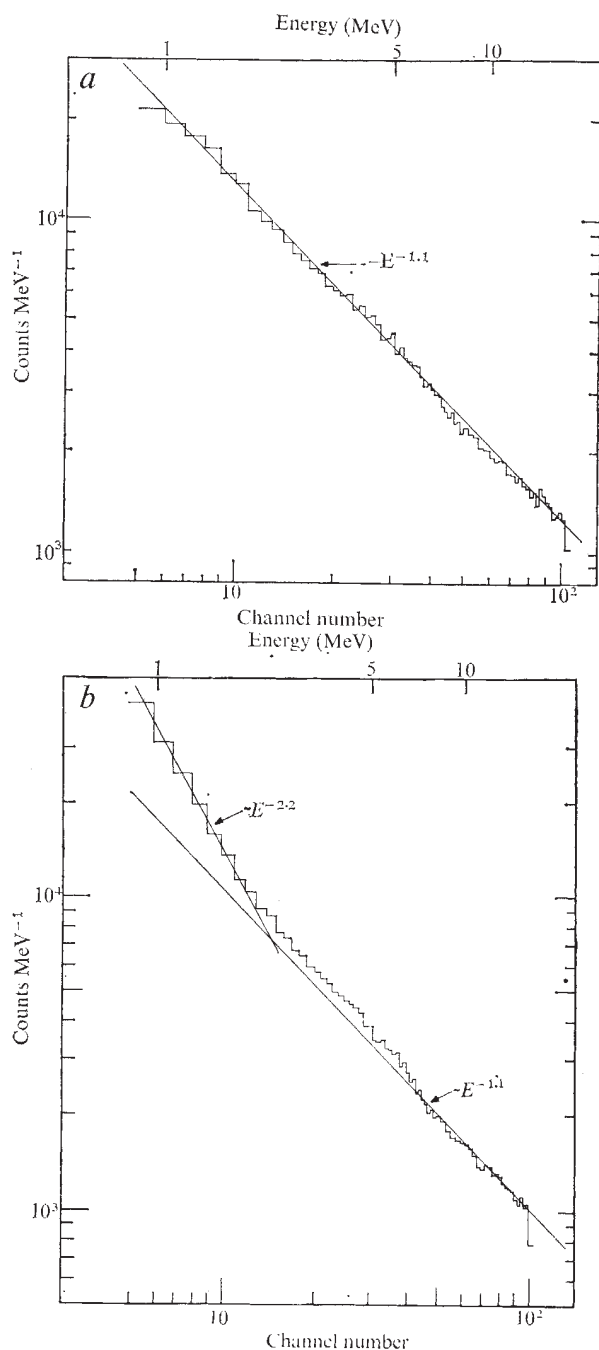


Fig. 2 *a*, Energy spectrum of the incident γ -ray flux during the period 1100–1230 UT on October 20, 1973. *b*, Energy spectrum of the incident γ -ray flux during the period 0900–1030 UT on October 20, 1973. 0.164 MeV per channel.

$L = 1.3$, we estimate that these γ rays are originating mainly from electrons with energies up to 10 MeV.

Increase in the precipitating particle flux at much lower energies (> 40 keV) has also been detected during the period in question by means of change in ionospheric conditions above São José dos Campos. Figure 3*a* and *b* show the ionograms taken at 0930 and 1000 UT on October 20 at Cachoeira Paulista located near São José dos Campos. Development of several well stratified ionospheric layers between 100 and 200 km is visible in the ionogram taken at 1000 UT. For comparison we have presented ionograms taken at the same hours on October 13 before the storm (Fig. 3*c*, *d*) and on October 26 after the storm (Fig. 3*e*, *f*).

Ionograms for October 7, corresponding to the first balloon flight, were not available. The presence of the sporadic *E* layer at 1000 UT on October 26 introduces some difficulty in interpreting

the echoes from the 100–200 km region. But, the nature of the traces obtained at these hours on October 13, and at 0930h on October 26, does suggest that the well defined layer between 100 and 200 km seen on October 20 results from the particle precipitation during the magnetic disturbance on this day.

The association between the increased precipitating flux of high energy particles and the geomagnetic activity has already been noted². The exact manner in which the geomagnetic field variations produce the increase electron precipitation is not clear. But short-period fluctuations (of the order of few minutes) in the geomagnetic field are believed to provide an effective mechanism (J. M. da C., N. O. T., and D. B. R., unpublished). Records of the total geomagnetic field intensity obtained at São José dos Campos shows that such short period fluctuations are present, and they could thus contribute significantly to the increased precipitation of electrons. An interesting feature

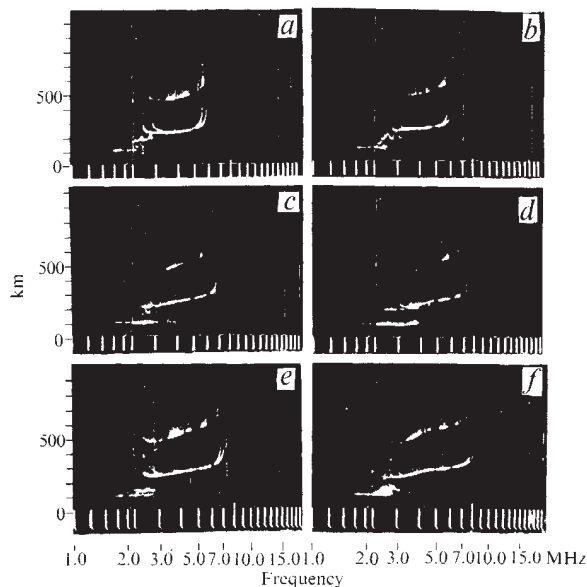


Fig. 3 The ionograms taken over Cachoeira Paulista on October 20. *a*, at 0930 UT; *b*, at 1000 UT. Also shown for comparison are the ionograms taken at the same hours on October 13, (*c* and *d*) and October 26 (*e* and *f*), which may be considered to be normal days. The frequency sweep in the ionograms is from 1 MHz to 20 MHz and the height markings are at 100 km intervals.

pertaining to the October 7 charged particle measurements is the apparent periodicity of about 30 min seen in the records at the ceiling altitude. There is also some suggestion of a periodicity in the curve of charged particle count rate for the flight of October 20. The corresponding curve for γ -ray flux also shows fluctuations but the periods seem to be shorter. The possible existence of a similar 30 min periodicity has been noted previously^{1,2} and related to the configuration of the magnetosphere over the anomaly region or to radial diffusion. More observations are needed to establish on more firm grounds the existence of such periodicities before we could attempt any detailed explanation for its origin.

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Observations of the Orion nebula at 100 μ m

WE observed the Orion nebula at coarse spectral resolution during flights on March 13 and 14, 1974, using a cooled grating spectrometer carried on NASA's Lear jet.

The instrument covered the spectral range from 80 to 125 μ m at a resolution of 8 μ m. It used a Ga:Ge photoconductive detector with an intrinsic long wavelength cut-off at approximately 130 μ m, and radiation shortward of 70 μ m was blocked by black polyethylene and a Yoshinaga filter. The field of view was roughly 5' square, which is large enough to include most of the brighter portions of the Orion 100 μ m source, according to D. A. Harper (unpublished).

To obtain a spectrum the grating was rotated to throw light of different wavelengths on to the detector. At each grating position measurements were made on and off the source, and the difference taken to be the source signal level. Several runs through the spectral bandpass were completed, taking steps of about 5 μ m, and a raw spectrum was obtained by averaging the values for a given grating position weighted according to the reciprocal of their variances.

Calibrations took two forms. The spectral calibration came from observations of the Moon which had to be taken on separate flights, since the elevation angles accessible to the Lear telescope are restricted and useful observing time is limited to about one hour per flight. But lunar spectra obtained on the mornings of March 11, 12 and 13 and the evening of March 7 are nearly identical. These spectra were used to correct for instrumental profile and atmospheric water vapour absorption, and for this purpose the moon was assumed to radiate as a 390 K blackbody¹.

The intensity calibration is based on a temperature for Venus (289 ± 20 K) from Armstrong *et al.*², and utilises Venus observations from March 11, 12 and 13.

Our Orion nebula spectrum corrected for instrument profile and atmospheric water vapour absorption is shown in Fig. 1 (left hand). Figure 1 (right hand) shows the lunar

spectrum obtained on the morning of March 13. The water vapour absorption feature at 100 μ m is relatively weak, and this is expected to be the case for all our observations, since they were made from several kilometres above the tropopause.

Superposed on our Orion data are 60 and 100 K blackbody curves diluted to give the right intensity, since the source does not fill our beam and may not be optically thin. Both curves fit the data, although grain emissivities of the form λ^{-n} give a better fit. Thus the best fits at 60 and 100 K are given by $n=1.7$ and 0.9 respectively, but in view of the low signal-to-noise ratio the difference from a blackbody is not statistically significant. Pure blackbody curves for temperatures below 40 K do not fit the data well, and the fit improves steadily for higher temperatures. Although our spectrum is somewhat steeper, our results are in good agreement with those of Erickson *et al.*³. Our flux calibrations also agree with the photometric data of Low and Aumann⁴ and Hoffmann, Frederick and Emery⁵. The error bars in Fig. 1 represent our standard deviation, and are derived from the standard deviations of the individual readings.

Our main conclusion is that our results are consistent with a thermal emission spectrum produced by cool interstellar grains radiating at a wavelength much longer than the grain size. If atomic or molecular lines dominate the 100 μ m flux, our spectral resolution is too coarse to detect them, but in any case there would have to be some conspiracy of lines to produce the apparently rather smooth spectrum we detect.

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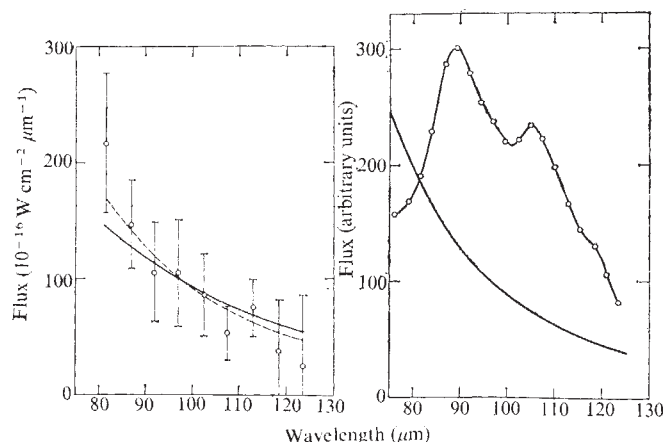


Fig. 1 Right-hand side, Orion nebula spectrum at 8 μ m resolution. The blackbody curves are diluted by factors of 83 for 100 K (---) and 2 for 60 K (—). Error bars are one standard deviation. Left-hand side, Calibration spectrum of the Moon (○—○), with superposed 390 K blackbody (—). Errors are too small to plot ($<2\%$).

Bathonian volcanicity and North Sea rifting

SEVERAL years ago^{1,2} we proposed that the Bathonian and Aptian fuller's earths of southern England, which are montmorillonitic clays, are true bentonites and, therefore, of volcanic origin (see also ref. 3). With the evidence⁴ of Bathonian volcanicity associated with rifting in the North Sea, we can now develop the story a stage further.

Two new publications^{4,5} outline the pre-Tertiary structure of the North Sea region in terms of a series of troughs separated by upstanding massifs or 'platforms'; we here adopt the more detailed reconstruction of Naylor *et al.*⁵ (Fig. 1). They have proposed a model in which thermal expansion of the crust and topmost mantle, perhaps consequent on the rise of a plume, leads to updoming and tensional collapse in the central region. This gives rise to trilete junctions at which three potential spreading ridges diverge. The Forties, Ekofisk and Northern North Sea (or Viking⁴) troughs form such a rift-rift-rift

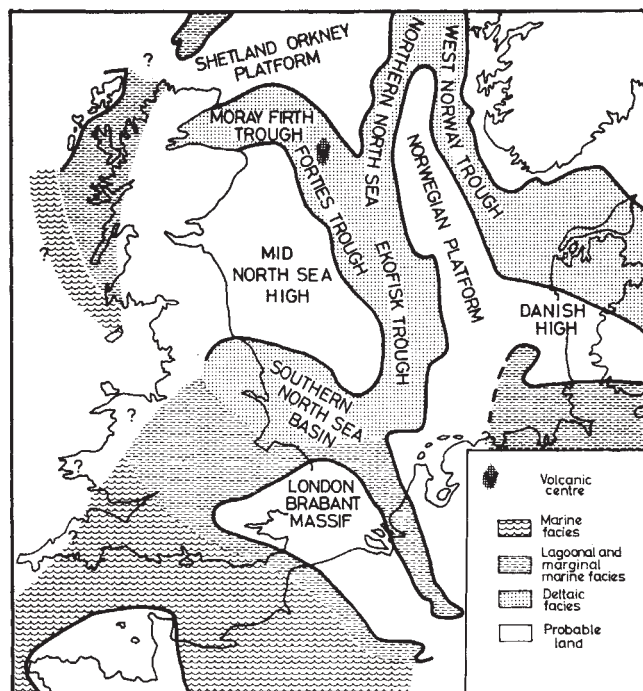


Fig. 1 Structural and palaeogeographic setting of Britain and adjacent areas during the Bathonian.

junction, and it seems to be highly significant that Bathonian volcanicity occurs at least approximately, at this junction⁴. The implied initiation of a major phase of updoming and rifting at this time can be supported on palaeogeographic grounds.

Considering the Jurassic geology of north-western Europe as a whole, it can be said that the gradual trend, in the early part of the Period, towards increasing marine conditions, with shallow seas progressively deepening and transgressing over the land, was reversed in the Middle Jurassic. All the way across from Britain to northern Germany the sedimentary facies tend to become more regressive from the Toarcian to the Bathonian, before more marine conditions became re-established in the Callovian. The sequence in Yorkshire, for instance, illustrates this well. Marine Lower Toarcian clays and shales pass up into shallower marine sands and ironstones in the Upper Toarcian and Aalenian, and then to mixed, shallow marine, and non-marine deltaic deposits in the Bajocian. The Upper Bajocian and Bathonian are wholly non-marine deltaic, and are replaced once more by marine deposits from the Lower Callovian upwards.

Viewed regionally, the area of greatest regressive tendency within the depositional regime can be identified as the central and northern North Sea (Fig. 1). In Yorkshire, marine influence during the Bajocian diminished northwards⁶, as it did in north-western Germany and southern Scandinavia⁷. In Andøye, northern Norway, the Middle Jurassic is wholly non-marine and deltaic⁸. The Bathonian of north-eastern Scotland is developed in deltaic facies, with no hint of marine deposits, whereas in western Scotland it is partly deltaic but mainly brackish-lagoonal and marginal marine. Across England and northern France, increasingly marine conditions can be inferred towards the south, and there is an increase in the proportion of calcareous deposits, signifying minimal influx of terrigenous sediment. In the Bathonian, for instance, the deposits are entirely deltaic in Yorkshire, mixed marginal marine and deltaic in the eastern Midlands, marginal marine in the southern Midlands and the Boulonnais and fully marine in Normandy and Dorset. A major source of coarse clastic sediments must be postulated in the North Sea region. Petrological evidence⁹ indicates this to be, in all probability, reworked late Palaeozoic sandstones, huge volumes of which were uplifted on the Mid-North Sea High and adjacent positive areas. (The

London-Brabant Massif does not seem to have been a major sediment source.) The finer sediments were probably derived partly from uplifted Triassic and Liassic rocks in the same areas.

Returning to the Bathonian fuller's earth of Somerset, the thickness of 1.5 m for a pure or almost pure montmorillonitic clay seems to be excessive for a single bentonite formed from ash carried some 450 miles from the volcanic centre. The most likely explanation is that a series of major eruptions took place during a restricted part of the Bathonian, at a time datable as *Retrocostatum* Zone, when normal terrigenous illite-kaolinite clay sedimentation was much reduced in the Bath area. A certain amount of reworking and ponding of ash deposits in small depressions would have enhanced the thickness locally. Montmorillonite is generally rare in British Jurassic clays and has not been found in beds older than Bathonian. We predict that bentonitic or montmorillonite-rich clays may be found over a wide region in late Bathonian deposits of central and northern England and Scotland; and they may provide a good stratigraphical marker horizon. On the basis of the bentonite mineralogy and chemistry, and the types of igneous rocks to be expected in early phases of continental rifting, the further prediction can be made that the lavas and/or tuffs will be basic to intermediate and alkaline in composition.

The initial rifting in the North Sea was not followed by sea-floor spreading, and therefore, the British Isles did not separate completely from the continent of Europe; only further trough subsidence accompanied by marginal faulting occurred, constituting what has been termed a 'Failed Arms System'⁵. This is in contrast to the subsequent Cretaceous rifting to the west and south-west of the British Isles, which also seems to have been associated both with volcanicity, judging from the Aptian bentonites and other evidence⁹, and upwarping of the continental crust, as marked by regional patterns of overstep and sedimentation¹⁰.

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Evidence of precursive compression for two deep earthquakes

DZIEWONSKI and Gilbert¹ have claimed that they can detect changes at a seismic source, starting 80 s before the origin time inferred from (impulsive) body wave arrival times. I believe, however, that their conclusion is almost certainly an artefact of their data analysis technique.

Dziewonski and Gilbert used the 'stacking and stripping' procedure⁴⁻⁶ to construct Fourier transforms of time

derivatives of the source time functions. That technique is applicable only at very long periods; as periods become shorter, lateral variations in dispersion become important. Dziewonski and Gilbert computed the transforms of the source rate functions only for periods of more than 78 s. Their procedure is exactly equivalent (if their reconstruction of the source rate transforms is correct) to taking the transforms of the source rate functions over the entire frequency range and then filtering the transforms by setting to zero all Fourier components with periods of less than 78 s, without changing the longer periods.

That type of filtering is 'non-causal.' If a delta function at $t=0$ is passed through this filter, energy will emerge from the filter at negative times, before the source energy arrives (Fig. 1). Dziewonski and Gilbert's procedure is de-

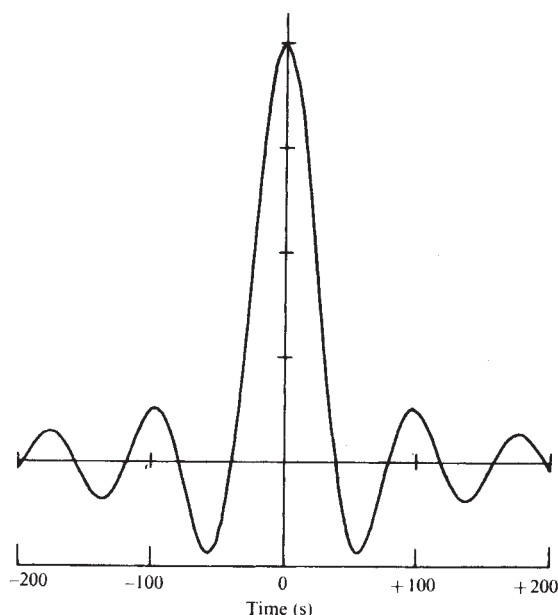


Fig. 1 Impulse response of the filtering procedure used by Dziewonski and Gilbert.

fensible only in cases where almost all of the source rate energy is concentrated in the ultra-low frequency band. But more than 85% of the energy of the (far field) source functions is contained, however, in the short periods, and so they cannot determine an accurate origin time from the free oscillation data set. If the Fourier transforms which they inverted to get their Fig. 2 had included all periods (instead of only the ultra long periods) it is quite likely that the time functions would all have been uniformly zero before the origin time.

The estimate that more than 85% of the spectral energy density was outside the frequency range considered by Dziewonski and Gilbert was derived by combining Aki's statistical scaling rule² with observational data. Aki's rule states that the far field displacement (or source moment rate) spectrum will be flat up to some 'corner frequency', above which amplitude falls proportionally to ω^{-2} . Because the energy density of the source moment rate is constant below the corner frequency, the total energy in a frequency band from zero to a cutoff below the corner frequency is proportional to ω . The results of Wyss and Molnar³ indicate that 0.1 Hz is a conservative estimate of corner frequency for the deep earthquakes considered by Dziewonski and Gilbert. Thus their cutoff frequency (0.013 Hz) eliminated well over 85% of the signal energy.

The conclusion that the hypocentral region began to undergo compression 80 s before the observed origin time is, therefore, unjustified.

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DRS DZIEWONSKI AND GILBERT REPLY: Although Geller discusses the problem of the rate of release of seismic energy, his criticism of our paper is concerned with our statement that the isotropic part of the moment rate tensor is precursive by 80 s. In this reply, we address ourselves to this precursive behaviour.

If $g(\omega)$ is the Fourier transform of $f(t)$, then $g(-\omega)$ is the Fourier transform of $f(-t)$. If $g(\omega)=g(-\omega)$ then $f(t)=f(-t)$. If $g(\omega)$ is an even function of frequency then $f(t)$ is an even function of time. If $f(t)$ is an even function of time it is precursive. The experimental evidence for the isotropic part of the moment rate tensor shows its cosine transform, an even function, to be large at low frequencies, and its sine transform, an odd function, to be small everywhere. Consequently, the isotropic part of the moment rate tensor has a Fourier transform that is an even function of frequency, from which follows that it is an even function of time, and, necessarily, precursive. The spectrum of the isotropic part of the moment rate tensor is very small for frequencies above 0.0048 Hz (0.03 s^{-1}). The precursor therefore should persist into negative time for about 105 s ($\pi/0.03 \text{ s}^{-1}$), which is in accord with our original, conservative estimate of 80 s.

Mr Geller shows a symmetric function of time that has a nearly flat cosine spectrum below 0.01313 Hz (0.0825 s^{-1}). It is nothing more than a low-passed delta function. The observed spectrum of the isotropic part of the moment rate tensor decays rapidly with increasing frequency, and is nearly zero above 0.0048 Hz (0.03 s^{-1}). Consequently it is not a low-passed delta function and must therefore be precursive.

The only way to invalidate this conclusion is to have the sine transform differ significantly from zero at very low frequencies, below 0.002 Hz (0.0125 s^{-1}).

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Correlation of strain and velocity during dilatancy

PRESENT earthquake mechanism models are predicated on the hypothesis of the formation of a dilatant zone in the region of the proposed break and the time history of physical rock properties that are a function of crack porosity. For example, field observations have shown drops in compressional wave velocity in earthquake areas prior to events. The anomaly persists for a time dependent on the source dimensions of the impending shock, and returns to normal prior to the event¹⁻⁴. Velocity measurements made during laboratory fracture tests have shown a decrease in compressional wave velocity above approximately 80% of the fracture stress^{5,6}. The effect is most pronounced normal to the direction of greatest compression. Similarly, as the fracture stress is approached the inelastic volumetric strain is the greatest⁷, indicating a definite correlation between velocity and inelastic volumetric strain.

Here we correlate spatial variations in compressional velocity with spatial variations in strain over the extended time interval required for the rock to fail under constant stress. A right circular cylinder of Westerly granite was statically loaded in uniaxial compression and velocity observed between the transducer pairs shown in Fig. 1. Coincident illumination and viewing holographic interferometry (refs 8, 9 and N. D. Meyer and H. S., unpublished) enabled us to observe continuously and interpret the entire strain field on the rock specimen.

At the start of the experiment a stress of $2.28 \times 10^7 \text{ kg m}^{-2}$ (2,240 bar) was applied for 24 h; then the load was incremented to be a constant $2.50 \times 10^7 \text{ kg m}^{-2}$ (2,450 bar) and held until failure. Strain (for the last 12 h only) is given in Fig. 1 as radial strain parallel to each of the velocity paths.

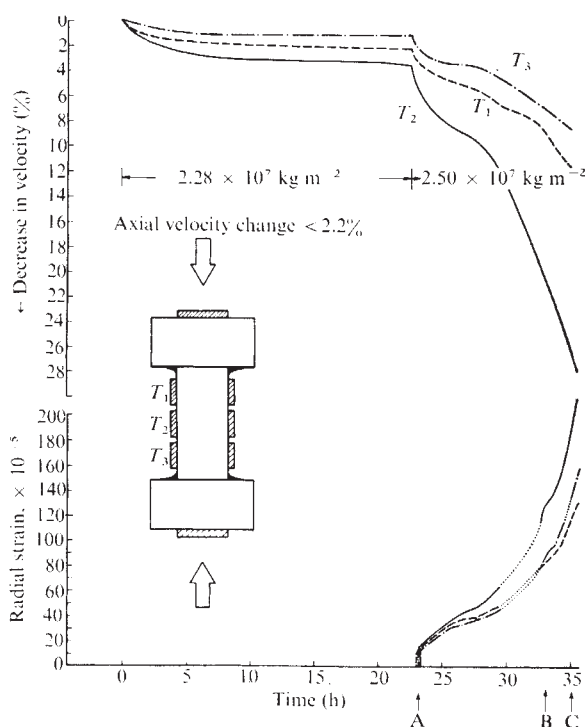


Fig. 1 Decrease in velocity (%) as a function of time over three paths, T_1 , T_2 and T_3 (see insert), for two creep episodes. The sample failed after 12 h at $2.50 \times 10^7 \text{ kg m}^{-2}$ (2,450 bar). The average radial strain over the same three paths for the second creep event is shown at the bottom of the diagram. The radial strain was calculated from 15 holograms which covered the entire last creep event. The first exposure of each hologram was taken immediately after the second exposure of the preceding one. This yields a complete strain history of the sample.

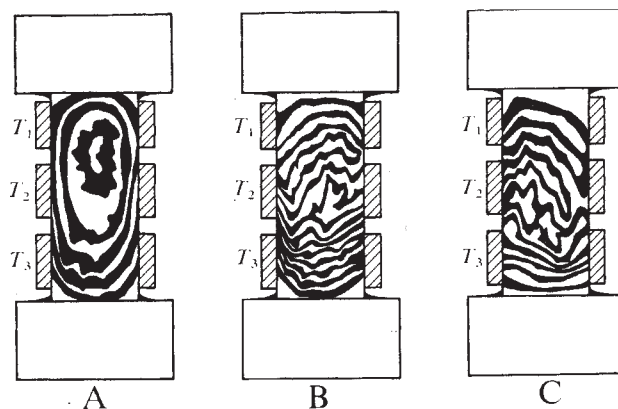


Fig. 2 Tracings of double exposure holograms at times A, B, and C as indicated in Fig. 1. Hologram A shows the effect of an axial stress increase from $2.28 \times 10^7 \text{ kg m}^{-2}$ (2,240 bar) to $2.50 \times 10^7 \text{ kg m}^{-2}$ (2,450 bar), B and C show the effects of creep during 44 min and 13 min intervals commencing 2.25 h and 0.25 h before failure, respectively.

The total axial strain, measured with a displacement transducer, was nearly linear over the last 12 h and amounted to 10^{-3} . This means that the sample was dilatant during the last episode and the inelastic volumetric strain curve will have the same general shape and characteristics as the radial strain depicted in Fig. 1.

Figure 2 shows three tracings of selected holograms, double exposed at times A, B, and C as shown in Fig. 1. Each hologram contains fringes which can be thought of as $0.3 \mu\text{m}$ contour lines in a topographic map of the surface change of the cylinder. The fringes result from the interference of two slightly different images projected into space.

Hologram A shows the change in surface displacement due to increasing the stress to $2.50 \times 10^7 \text{ kg m}^{-2}$ (2,450 bar). The contours are smooth and form easily identifiable ellipses. The strain is concentrated at the top of the sample and may be characterised as a broad asymmetric bulge along the axis of the sample. The strain field in hologram B taken eight hours after A is much more complex. The fringes are irregular, the strain concentration has shifted toward the bottom of the sample, and the strain ellipse strikes NE. The distortion of fringes is attributed to surface crazing prior to spallation. The contour fringes are much more closely spaced than in A, indicating a larger strain accumulation. The interval between exposures was 44 min. Hologram C was completed 15 min prior to failure; the interval between exposures was 13 min. The greatest strain is still concentrated in the lower half of the sample, and the fringes are distorted, but the strain ellipse now strikes NW. This suggests that the strain field in the sample is not only inhomogeneous but regions of peak strain migrate through the sample. From an examination of all 15 holograms, three such changes in orientation were noted. It seems that two incipient conjugate faults form prior to failure with the strain accumulating on each independently.

The transverse velocity decreased rapidly following the stress increase at approximately 24 h. The slight change in slope at 28 h was then followed by a precipitous plunge toward failure. The radial strain data in Fig. 1 were obtained from holograms along three lines corresponding to the transverse velocity paths. The similarities between the velocity and strain histories are apparent. First, the location of greatest velocity falloff corresponds to the region of largest inelastic strain. Second, there is a definite correlation in the shape of both sets of curves. The transients in all six curves persist for approximately the same period, and the accelerated segments, prior to failure (tertiary creep), all commence at the same time.

So there is a strong correlation between the local inelastic strain and the velocity. The large variations in velocity over

several centimetres distance were unexpected. In addition, the resolution of the holograms presented here shows holographic interferometry contributes significantly in unravelling the local changes in inelastic strain that precede failure.

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Tholeiite, alkali basalt and ascent velocity

ALKALI basalts occur in a great variety of geological environments. It would, therefore, be surprising if only one mechanism were responsible for all occurrences, but we attempt here to explain some features of alkali basalt volcanism which are not readily understood in terms of the deep origin theory.

Engel *et al.*¹ concluded that the great majority of rocks from the ocean floor were tholeiitic basalts, and that the occurrence of oceanic alkali basalts was restricted to the tops of volcanic islands and sea mounts. This generalisation is still largely valid, though, apparently, a greater variety of rocks types are found in the ocean basins^{2,3} than was admitted by Engel *et al.*¹

The pillow basalts at the top of layer 2 of the oceanic crust are believed to comprise mainly tholeiite, produced at active spreading ridges. The most important parts of many oceanic islands also seem to be tholeiitic; this has been observed for Hawaii², Reunion, and several other islands. Plateau basalts such as the Columbia River Basalts and the Deccan Basalts, are predominantly tholeiitic, as are gabbroic layered intrusions such as Skaergaard and Muskox.

Alkali basalts are frequently produced during the last stage of oceanic volcanism as has been described for Hawaii⁴ and Reunion Island⁵, but there are some oceanic volcanic islands, such as the Canary Islands, which seems to be made up mostly of alkali basalts⁶. Alkali basalts also occur at the ocean floor, near ridge axes³ and transform faults⁷. The Red Sea^{8,9} and the Afar region¹⁰, seem to be underlain by alkali basalt or tholeiite with definite alkaline characteristics. Alkali basalts are typical of the African rift zones.

Alkali basalts are frequently associated with the tholeiitic flood basalts as is the case in Britain and Greenland¹¹. Marginal oceanic basins seem to contain tholeiites which tend to have alkaline characteristics¹².

Laboratory experiments^{13,17} on the origin of alkali basalts and tholeiites, have been interpreted to indicate that tholeiitic basalts have been formed by a relatively large degree

(25%) of partial fusion of a peridotitic parent material at a shallow depth in the upper mantle; and alkali basalts are thought to come from a great depth in the upper mantle. Geochemical arguments based on the contents of trace elements in alkali basalts and their presumed peridotitic parent material, have led to the conclusion that alkali basalts can only be produced by small degrees of partial fusion¹⁸.

We propose an alternative model. Because of the poor thermal conductivity of silicates, vertical movements in the mantle have been considered to be, in general, adiabatic. Thus, the rate at which the temperature changes in a mantle diapir is, to a first approximation, given by the adiabatic gradient: $0.3^{\circ}\text{--}0.5^{\circ}\text{C km}^{-1}$. If the diapir rises to the surface and is already at a temperature close to its solidus temperature, partial melting can be anticipated, because the solidus temperature of potential upper mantle materials varies with depth by about $4^{\circ}\text{C km}^{-1}$ (ref. 14). If, however, the temperature contrast between the diapir material and the surrounding upper mantle becomes large, and/or when the upward vertical velocity of the moving diapir becomes small, the diapir may cool faster than adiabatic cooling, because heat transport by conduction may then become significant as compared with heat transport by mass transfer.

Numerical calculations^{19,20} indicate that at depths of more than 25 km, and velocities of less than 1 cm yr^{-1} , the rate of cooling of a massive, upward moving current of silicate crystal mush, becomes greater than the variation of the basalt solidus temperature with depth. When conductive cooling becomes important relative to adiabatic cooling in rising mantle diapirs, the amount of partial fusion of the diapir will diminish; thus, the liquid produced because of decompression in the diapir will tend to be more alkaline than would be the case for a rapidly rising diapir, in which conductive cooling would be negligible.

In the case of Hawaii, there is evidence that tholeiite is generated at a depth below 60 km (ref. 21) by about 30% partial infusion of a herzolitic upper mantle³. Seismic evidence²¹ suggests upward lava velocities considerably in excess of 1 cm yr^{-1} . If, however, the lava moved upward more slowly, as may occur during the waning stage of volcanic activity, then it may start to precipitate before reaching the surface and will then tend to develop alkaline characteristics which will be further accentuated by contamination with wall rock. Contamination is more serious in slowly moving lavas.

The theory of deep origin for alkali basalts, as applied to Hawaii, suggests that alkali basalt also originates at depth in excess of 60 km, below the oceanic lithosphere, in the asthenosphere or even deeper. The prolonged alkali basaltic activity of islands in the Atlantic, such as the Western Canary Islands, is therefore difficult to understand. Likewise, the apparent total absence of tholeiites on several islands in the Atlantic is not readily explained by the theory of deep origin for alkaline basalts.

Another situation in which the rate of ascent may determine the type of basalt, is the case of very slow seafloor spreading. Basalt erupted at an active spreading centre is thought to be generated by partial melting, as a result of pressure decrease, in upward moving mantle material coming from the low velocity zone^{19,20,22}. Low spreading velocities imply low rates of ascent underneath the ridge centre. This situation has been modelled quantitatively, and it was found that for vertical velocities of $1\text{--}8\text{ cm yr}^{-1}$ the composition of ridge basalt does not depend on the rate of ascent²⁰. But for vertical velocities of less than 1 cm yr^{-1} the basalt composition became more alkali rich than tholeiite.

Thus, it can be understood why, in regions characterised by very low plate velocities, alkali basalts or tholeiites with definite alkaline tendencies are frequently found. An example is the Red Sea⁸ which has a plate velocity of less than 1 cm yr^{-1} . There are alkali basalts at the Goringe Bank in the Atlantic²³. This location is situated in the magnetic

quiet zone which itself possibly results from a very slow rate of seafloor spreading²⁴.

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Ubiquitous trisulphur radical ion S_3^-

THE colour of blue ultramarine and the deep blue colour of alkali polysulphides in electron pair donor solvents have been attributed to the disulphur radical ion, S_2^- (ref. 1). That assignment is erroneous and I here establish the correct identity of the species, emphasise the variety of situations in which it is encountered and point out its possible role as an intermediate, in transformations involving elemental sulphur, or sulphide ions.

A blue colour develops when sulphur is heated with water and traces of some basic salt^{2,3}. Blue solutions are also formed by sulphur in alkali halide melts⁴, in liquid potassium thiocyanate above 300°C (refs 5 and 6), by alkali polysulphides in basic solvents^{7–12}, by electrochemical reduction of S_8 in dimethyl sulphoxide^{13–15} or LiCl–KCl eutectics^{16,17}, and in the electrochemical oxidation of alkali metal polysulphides in eutectics¹⁸. The blue species is also present (as a minor component) in

dilute solutions of sulphur in ethylenediamine^{19–21} and in the mineral lapis lazuli^{22,23} and it is formed from proteins containing fully combined cystine, for example, insulin in liquid ammonia²⁴, from dimethylamine and elemental sulphur in hexamethylphosphoramide¹¹, and in acetone solutions of sulphur containing potassium hydroxide²⁵.

The blue species is characterised by a visible absorption band at 610–620 nm, and it can be attributed to the trisulphur radical anion, S_3^- (refs 10, 12, and 15). It is well established that S_3^- (in the solid state) has an optical absorption at about 610 nm, which is associated with a Raman band at 534 cm^{-1} , whereas S_2^- shows a Raman band at 594 cm^{-1} associated with an optical absorption band at about 400 nm (refs 26–28). Furthermore, S_3^- exhibits a characteristic infrared band at 580 cm^{-1} (refs 10, 11, and 26), (antisymmetric stretching mode), whereas S_2^- is, as expected, infrared inactive. The electron spin resonance (e.s.r.) spectra of S_3^- (ref. 29) and S_2^- (ref. 30) are also well characterised, and the technique has been used to detect S_3^- on the surface of the catalyst when elemental sulphur reacts with partially hydroxylated magnesium oxide at 400°C (ref. 31).

Although the existence of catenated species is a general feature of sulphur chemistry, and probably results from the relatively high σ -bond energy, compared with π -bond energy, for sulphur³⁰, the formation of S_3^- in preference to other sulphur radical ions is not readily explained. Like other 19e triatomic molecules for example, O_3^- and ClO_2^- (refs 32, 33) S_3^- will have C_{2v} symmetry with $SSS < 120^\circ$. The odd electron will occupy a molecular orbital which is S–S antibonding, but bonding with respect to terminal sulphur atoms³⁴. The possibility of $S \cdots S$ interactions between terminal sulphur atoms must, therefore, be considered. For the S_3^{2-} ion, of known structure¹, the separation would be 3.37 Å. According to the Walsh rules for triatomic molecules³⁴, the SSS angle would increase slightly for S_3^- whereas the S–S distance should decrease compared with S_3^{2-} , so that the $S \cdots S$ separation in S_3^- can be expected to be similar to that in S_3^{2-} . Although this value is lower than the sum of the Van der Waal's ratio for sulphur (3.70 Å), it is considerably larger than the S–S intraring interactions in S_8^{2+} [$d(S_8-S_7) = 2.86$ Å]³⁵ and about the same as the intraring distance in S_8 [$d(S_1-S_8) = 3.33$ Å]³⁶ which is not regarded as implying a significant interaction³⁷. It is noteworthy, however, that the dithionite ion, $S_2O_4^{2-}$, with an unusually long S–S distance (2.39 Å)³⁸ dissociates in oxygen-free aqueous solution to give the SO_2^- radical ion³⁹, isoelectronic with S_3^- .

The probable significance of the S_3^- radical ion (and other sulphur radical anions) in a wide variety of situations should be recognised. For example, the formation of S_3^- in superheated water must be considered in a discussion of sulphur transformations which occur in geothermal springs⁴⁰. The discovery that sulphur radical ions are the electroactive species in the electrochemical oxidation of polysulphides is significant to battery technology, particularly for the application of Na/S⁰ or Li/S⁰ couples in secondary battery systems¹⁸. The formation of sulphur radical ions on the catalyst surface should be taken into account in any discussion of the mechanism of desulphurisation reactions and the reduction of SO_2 over metal oxides³¹. The well known Willgerodt–Kindler reaction provides an example of the catalytic effect of amines on the reactions of elemental sulphur⁴¹ which undoubtedly involves the formation of polysulphides⁴², and thus, sulphur radical ions^{15,43}.

The common feature of the wide variety of conditions in which the S_3^- ion is formed is the existence of conditions in which polysulphide ions, S_x^{2-} , are either present or can be formed easily. The trisulphur radical ion can then be produced from polysulphides either through dissociation ($x = 6$) or by disproportionation ($2 < x < 6$). As electrochemical studies^{13–18} have shown that the reduction $S^0 \rightarrow S^{2-}$, or the oxidation $S^{2-} \rightarrow S^0$, is not a single step process but occurs through polysulphide ions, the intermediacy of sulphur radical anions, for example, S_3^- in, for instance, the biological oxidation of sulphide, is a real possibility⁴⁴. Indeed, sulphur radical ions

have been invoked to explain the oxidation, by aqueous polysulphide solutions, of aryl methyl compounds to the corresponding carboxylic acids⁴⁵.

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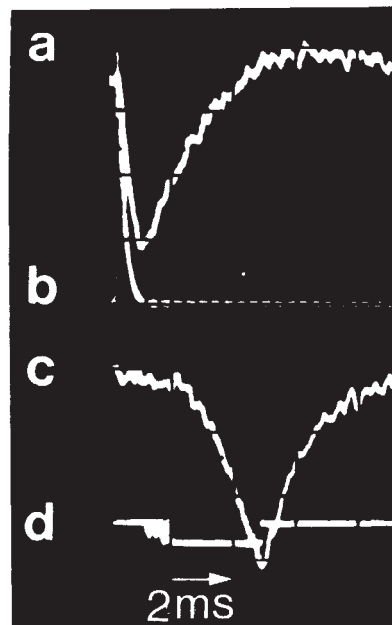


Fig. 1 Birefringence induced in Bentonite sols, at a wavelength of 488 nm. a, Laser-induced birefringence response; b, YAG laser ($\lambda=1.06 \mu\text{m}$) 'fixed Q' pulse of approximately 8 kW maximum power; c, electrically induced birefringence response; d, a direct current electric pulse, of amplitude 31 V cm^{-1} . Time scale, 2 ms per division; a linear detection system¹¹ was used. For b and d the same incident light intensity was used; the amplitudes of the responses are directly comparable and actually represent decreases in intensity and, thus, a negative birefringence. The sol concentration was $1.3 \times 10^{-3} \text{ g ml}^{-1}$.

effects accompany particle orientation rather than local heating or refractive index changes, and that they therefore indicate the foundation of a new method for characterising macromolecules.

Many molecules and particles have anisotropic optical properties. When suspended in a liquid they adopt random orientations and the overall medium is optically isotropic, until some external force field—electric, magnetic, acoustic, and so on—is applied to the suspension. Then, the anisotropic properties of the individual particles become partially impressed on the bulk medium, as the suspended particles become aligned.

Changes in the optical properties (scattered intensity¹, birefringence², dichroism², optical activity³) when pulsed electric fields are applied, have been studied often in macromolecular suspensions. The pulsed method enables molecular relaxations to be studied, whilst electric fields lead to information on the permanent dipole moments and electric polarisabilities of the suspended particles. These 'electro-optical' methods have proved particularly valuable in the study of macromolecules, because of their relatively large permanent and induced dipole moments.

It has been predicted⁴ and shown experimentally⁵ that the electric field associated with a suitably highly powered pulsed laser could provide the force field in the case of simple liquids. Similar effects have been anticipated⁶, but not until now realised experimentally for suspensions of macromolecules.

We assembled a conventional birefringence apparatus, with an Argon Ion, low power, continuous working laser which provided the probe light beam at a wavelength, λ , of 488 nm. The 'orienting' beam was obtained from a Nd^{3+} YAG laser working at a wavelength of $1.06 \mu\text{m}$. At optical frequencies, only the electronic contributions to the electrical polarisability are effective, so that a high field is required before a suitable dipole moment can be induced in the molecules. Relatively long orientation times, from $10 \mu\text{s}$ to 1 ms , are required for the rotation of macromolecules⁷. To satisfy both of these requirements, the YAG laser was operated in a 'fixed Q' manner, in which the gain of the cavity was fixed and the laser was driven at a high power for a short, prefixed time; in this case approximately

Laser-induced orientation in macromolecular suspensions

We report here the first realisation of laser-induced changes in the orientation of macromolecules and particles in a liquid medium. We give preliminary data on the induced birefringence of two, very dilute, macromolecular suspensions; and we show that the

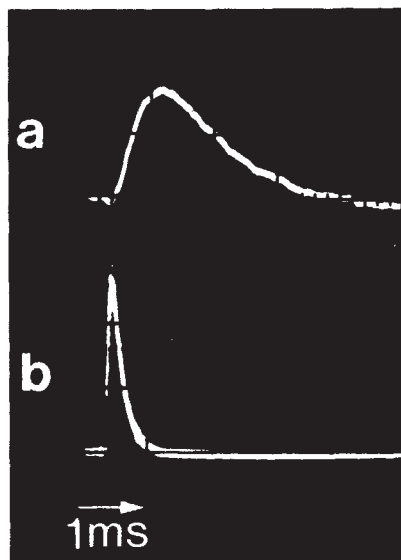


Fig. 2 Laser-induced birefringence of a solution of TRV at a wavelength of 488 nm. a, Birefringence response from TRV. Time scale, 1 ms per division. The response is an increase in light level and, therefore, a positive birefringence effect. TRV of 3×10^{-3} g ml $^{-1}$ was studied in M/60 Sorinsens phosphate buffer at pH = 7.0. b, YAG laser 'fixed Q' pulse of approximately 8 kW maximum power.

200 μ s. Conventional Q switching produced pulses which were much too fast. The power of the 'fixed Q' beam was monitored through a calibrated photodiode and an approximate equivalent field strength, E , was calculated using the Poynting vector equations⁸. Precise values for E were not obtained, because of the complex shape of the pulse with time. It was important to operate the YAG laser in the single transverse mode, for only then was the electric vector unidirectional throughout the beam cross-section. The beam was unfocused and polarised at 45° to the azimuth of the initial polariser of the birefringence optics. Using suitable dielectric coated mirrors, which transmitted the probe blue light but totally reflected the YAG infrared radiation, the orienting beam was made to travel the length of the sample cell together with the probe beam, thereby allowing a large optical cell length.

Aqueous suspensions of Wyoming sodium bentonite, a disk-like clay mineral⁹, and a solution of tobacco rattle virus (TRV), a rod-like virus¹⁰, were studied. Figures 1a and 2a are typical of the transient birefringence traces observed. In each case the applied laser pulse is shown (Figs 1b and 2b). In order to verify that these effects arose from particle orientation, two further experiments were performed. In the case of the bentonite sol, a pulsed d.c. electric field (Fig. 1d), of suitable amplitude and duration, was applied after the laser beam. The field strength was chosen to give a similar birefringence response

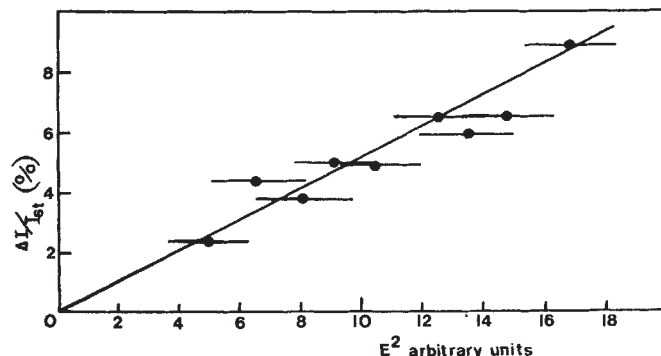


Fig. 3 Change of maximum birefringence with the square of the effective field strength, E , of the YAG laser beam. With linear detection, the light intensity change, ΔI , is directly proportional to the birefringence, and I_0 represents the residual transmitted intensity. Data for TRV.

(Fig. 1c) to that induced by the laser beam; the transient decay from both the electrically and the laser-induced effects have the same time constant, within experimental uncertainty. When interpreted as a molecular relaxation time, τ , in the conventional manner for electric birefringence data, a value of $\tau = 1.9$ (± 0.2) ms was obtained. That is typical of the rotational relaxation time of that material¹². With TRV, the laser-induced birefringence led to a value of $\tau = 1.0$ (± 0.1) ms, which also agrees with the value reported elsewhere¹³.

Rotational theory also predicts that² the birefringence increases as the square of the electric field. Measurements were made as a function of the laser power and are presented in Fig. 3 in terms of the strength of the equivalent electric field; Fig. 3 shows the predicted quadratic dependence on E .

Future experiments will be made to quantify accurately the amplitude of the induced effects so that the electronic contribution to the polarisability can be isolated. Apart from that, and the close association with nonlinear optical effects, particle sizes can also be evaluated¹⁴ from τ . We conclude, therefore, that these preliminary studies could lead both to a series of experiments which characterise macromolecules, and to a greater understanding of their electrical and optical properties.

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Total reflection as derived from statistical scattering and coherent scattering theories

IN the past ten years there has been much investigation of the interesting phenomenon of microscopic bubbles in liquids, particularly liquid helium, which are maintained by the presence of a light particle, such as an electron¹ or positron². In both cases, with liquid helium the bubble is believed to arise from an effective repulsive potential between bulk liquid and the light particle. Coopersmith³ derived an expression for this effective potential by calculating the free energy of a light particle moving in a space having randomly distributed hard spheres.

The hard spheres move as in a classical ideal gas and statistical equilibrium between light particle and gas is assumed. Only s-wave scattering was considered. The mean free energy of the light particle is found to have a minimum energy which is due to space-limitation of its motion. The interaction free energy was shown rigorously to be

$$E_F = 2\pi n a_s h^2 / m \quad (1)$$

due to single scattering, all higher order multiple scattering effects being zero. In equation (1) n is the density of scattering centres, a_s the scattering length and m the light particle mass. Thus a particle of mass m striking the surface of a liquid having n molecules per unit volume from outside would require a kinetic energy at least $k^2\hbar^2/2m=E_F$ in order not to be totally reflected from the liquid surface. Therefore, the critical wave number for total reflection at normal incidence is given by

$$k^2 = 4\pi n a_s \quad (2)$$

Eighteen years earlier, Goldberger and Seitz⁴ investigated coherent scattering from randomly distributed scattering centres, and produced results which have, for example, been applied to neutron refraction at liquid surfaces. According to their theory a particle of mass m will be totally externally reflected when normally incident upon a liquid surface unless

$$k^2 \geq 2n(\pi\sigma_s)^{1/2} \quad (3)$$

which agrees with equation (2) if we put $\sigma_s = 4\pi a_s^2$ and take the positive square root, indicating repulsion.

So these two strikingly different approaches yield the same result in the low energy limit, for s -wave scattering.

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Measurements of K, Rb, U, Sr and Pb in diamonds containing inclusions

KNOWLEDGE of the Pb and Sr isotopic compositions of syngenetic inclusions in diamonds is important for several reasons. As diamonds probably crystallised at depths of more than 100 km, the isotopic data may provide constraints on the mode of the geochemical evolution of the Earth. It may also be possible to test assumed U/Pb ratios ($U/Pb = \mu$) for the mantle and to estimate the age of particular diamonds. Diamonds are particularly appropriate for these studies because, in contrast to kimberlites and their associated nodules, any contamination by crustal material can be overcome by washing in acid.

Here we report preliminary measurements of the K, Rb, Sr, U and Pb concentrations in diamonds. As far as we know the only other reported measurements are by Fesq *et al.*¹ who used instrumental neutron activation analysis to determine the concentrations of Sr, Rb and K. They found that these elements are far more abundant in diamonds with visible inclusions than in those apparently free of inclusions.

We investigated two batches of diamonds from the Premier Mine, South Africa, one containing green (mainly diopside) and the other black (mainly sulphide) inclusions. The 'green' diamonds (22 stones, weighing 0.2674 g) were analysed for Sr, Rb and K, and the 'black' diamonds (27 stones, weighing 0.3188 g) for Pb and U. The diamonds were small, 1–2 mm in size (in general, inclusions are of roughly constant size, so for a given weight of diamonds the percentage occupied by the inclusion is highest in small stones). The diamonds were colourless or brown, of good crystal morphology and free from surface fractures.

Diopside is a fairly common inclusion in Premier Mine diamonds, occurring as pale green euhedral or anhedral crystals. The diopsides chosen were free from associated black material² although in some cases internal fractures were present. The sulphide occurrences in diamond are more varied³, and sulphides occurring in internal rosette fractures associated with colourless inclusions, and as discrete sulphide inclusions were chosen. The sulphides probably consist mainly of pyrrhotite², but the chemistry was not determined. They were distinguished from graphite by colour and lustre³. The percentage by weight that the inclusions occupied within both batches was roughly estimated as 0.1%.

The Premier pipe is older than $1,115 \pm 15$ Myr and younger than $1,954 \pm 30$ Myr (refs 4 and 5). It contains at least four distinct intrusions of kimberlite with ages that may range from 1,200–1,400 Myr, but it could not be ascertained to which type our diamonds belonged. The best dated type of kimberlite (Type 1) has a Rb/Sr age of $1,250 \pm 20$ Myr (H. L. A. unpublished), so we assumed that age in our work, but the assumption of any age in the range indicated would not affect significantly our tentative conclusions.

For each experiment we used only 10 g of reagent prepared by triple distillation in quartz under sub-boiling point conditions (in the case of H_2O and HCl); HF and HNO_3 were prepared as in ref. 6.

The diamonds were cleaned by prolonged and repeated heating and ultrasonic agitation in HF , $HClO_4$ and water, and then burnt over a period of about 15 min in a clean, covered quartz crucible at $\sim 850^\circ C$, in a flow of high purity filtered oxygen. A preliminary experiment using a platinum crucible gave a Pb blank much higher than the quartz; but even the quartz (or possibly the oxygen) still constitutes a serious source of Pb contamination. The crucible, about 15 cm long, had a water-cooled ring near the top to condense volatilised molecules.

The residue was transferred to a teflon autoclave⁷ using dilute acid. After HF was added it was heated at $200^\circ C$ for 6 h. After evaporation the residue dissolved readily in dilute HCl . This solution was then aliquoted for three separate mass spectrometer analyses: isotopic composition (of Sr and Pb); concentration of K, Rb (jointly) and U; and concentration of Sr and Pb.

Concentrations were determined by isotope dilution. Isotopic tracers of high purity (>99%) were used in all

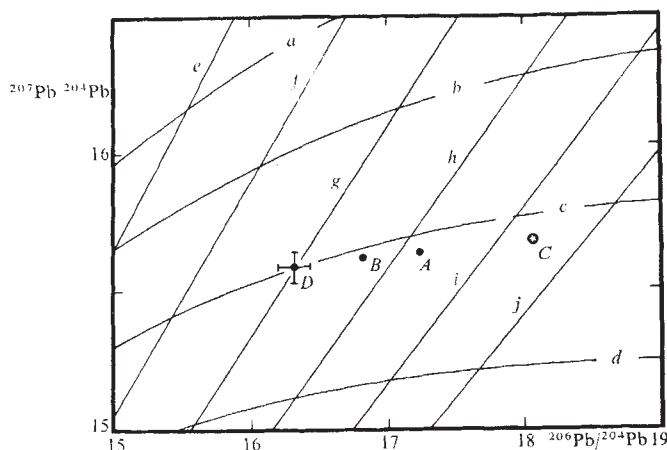


Fig. 1 $^{207}Pb/^{204}Pb$ and $^{206}Pb/^{204}Pb$ data plotted on a lead growth diagram. a , $\mu = 11$; b , $\mu = 10$; c , $\mu = 9$; d , $\mu = 8$; e , $T = 2,500$ Myr; f , $T = 2,000$ Myr; g , $T = 1,500$ Myr; h , $T = 1,000$ Myr; i , $T = 500$ Myr; j , $T = 0$. A , Experimental data corrected only for mass spectrometer fractionation; B , results from correction for the blank, the isotopic composition of which is indicated by C ; D , results from the additional correction for radiogenic Pb. Parameters used: $a_0 = 9.56$; $b_0 = 10.42$; $\lambda^{238} = 0.1537 \times 10^{-9} \text{ yr}^{-1}$; $\lambda^{235} = 0.9722 \times 10^{-9} \text{ yr}^{-1}$.

Table 1 Results on inclusions in Premier diamonds

	K (ng)	Rb (ng)	Sr (ng)	K/Rb	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}$	U (ng)	Pb (ng)	$^{238}\text{U}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$
Reagent blank (2 g HNO ₃ + 2 g HF + 6 g H ₂ O)	20.9	0.102	0.398				0.239	0.62				
First full blank on method (10 g reagent)	65.0	0.128	0.339				0.402	12.14				
Final full blank on method (10 g reagent)	16.3	0.106	0.718				0.367	13.60				
Average of blanks on method	40.7	0.117	0.529				0.385	12.87				
'Green' pyroxene inclusions	599.4	2.866	59.89			0.7053* ±0.0007						
% Blank	6.8	4.1	0.88									
'Black' sulphide inclusions							1.529	40.22		17.24†	15.64†	37.05†
% Blank							25.2	32.0		±0.06	±0.05	±0.20
Inclusion results — blank on method	558.7	2.749	59.36	203.2	0.135	0.7053‡ ±0.0007	1.144	27.35	2.473	16.84§ ±0.06	15.62§ ±0.05	36.24§ ±0.20
Age-corrected isotopic ratios¶						±0.7029 ±0.0007				16.32 ±0.12	15.58 ±0.06	

* Normalised to $^{86}\text{Sr}/^{86}\text{Sr} = 8.375$.

† Corrected for mass-spectrometer fractionation to NBS 981 standard.

‡ Corrected for blank $^{87}\text{Sr}/^{86}\text{Sr} = 0.7090$.

§ Using measured blank lead isotopic composition $206/204 = 18.08$; $207/204 = 15.68$; $208/204 = 38.77$.

¶ Using age of Premier pipe of 1,250 Myr; error for Pb ratios arbitrarily increased to take account of uncertainty in U/Pb ratio; $\lambda^{86} = 1.39 \times 10^{-11} \text{ yr}^{-1}$.

All quoted errors are estimated 1σ values.

cases except U, and the sensitivity was accordingly good for all elements except U. The accuracy of the concentration data is limited in all cases (again except U) by the blank levels and not by the precision of the mass spectrometer measurements. Potassium, rubidium and lead were run on single filaments by the silica-gel method, and Sr and U were run on double filaments. The decision to make no chemical separations of the elements under consideration had deleterious effects in both the Sr and Pb isotopic composition analyses. With Sr, Rb interference ($^{87}\text{Rb}/^{87}\text{Sr} \sim 2\%$) was a problem, but correction using $^{86}\text{Rb}/^{87}\text{Rb} = 2.600$ was considered reliable as no samples enriched in ^{87}Rb have even been measured on the mass spectrometer in question. With Pb, although test runs with pure, 50 ng, Pb samples gave satisfactory ion beams, the diamond sample yielded only a small beam ($^{204}\text{Pb} \sim 1-3 \text{ mV}$), and this severely limited the precision.

Table 1 shows the results obtained. The blanks of Sr, K and Rb (both on the reagents and on the full procedure) are of an acceptably low level, but the blanks of U and Pb amounted to 25% and 32% of the sample, respectively. The U tracer used is not sufficiently sensitive for such low level work, and the blank levels may well have been overestimated. Only in the case of Pb is the full procedure blank significantly greater than the reagent blank. The most significant result is that ~ 30 and 60 ng of Pb and Sr,

respectively, are contained in the relatively modest quantities of diamonds used. On the evidence of Fesq *et al.*¹ and our own unreported data from a preliminary experiment on a batch of diamonds with only graphite inclusions, we believe that this Pb and Sr is contained in the inclusions; using the very rough abundance estimates of the inclusions, the concentrations are $\sim 100 \text{ p.p.m.}$ of Pb in the sulphides and $\sim 200 \text{ p.p.m.}$ of Sr in the diopsides. But even if the Pb and Sr is contained in the diamond structure rather than in the inclusions, our conclusions relating to the isotopic data will not be affected. The K/Rb ratio of the diopside inclusions is ~ 200 , a similar value to that obtained for peridotite nodules and their constituent diopsides⁸.

The Pb and Sr isotopic ratios were corrected in several ways. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio was normalised to a $^{86}\text{Sr}/^{86}\text{Sr}$ ratio of 8.375; if the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the contamination Sr was 0.7090, the blank correction is negligible; correction for the radiogenic component was made on the basis of the measured Rb/Sr ratio and an age of 1,250 Myr. The $^{207}\text{Pb}/^{204}\text{Pb}$ and $^{206}\text{Pb}/^{204}\text{Pb}$ ratios were arbitrarily corrected for fractionation by comparing measured and accepted values on the NBS 981 standard Pb. Correction for the radiogenic Pb was made using the measured U/Pb ratio, and because of the large uncertainty in this ratio, the error on the isotopic ratios has been increased arbitrarily. The influence of the isotopic composition of the contaminating Pb is in

Table 2 Rb, Sr and $^{87}\text{Sr}/^{86}\text{Sr}$ values for Premier carbonatites

Sample	Rb (p.p.m.)	Sr (p.p.m.)	$^{87}\text{Sr}/^{86}\text{Sr}$	Remarks
PC/B	0.32	1,042	0.7025	High Mg-brucite
PC/2	1.48	838	0.7032	Si-rich kimberlitic carbonatite
PC/3	0.85	1,579	0.7044	A complex rock, predominantly calcite

$^{87}\text{Sr}/^{86}\text{Sr}$ corrected to $^{86}\text{Sr}/^{86}\text{Sr} = 8.375$; accuracy in $^{87}\text{Sr}/^{86}\text{Sr}$ estimated as ± 0.0003 . Using an age of 1,250 Myr the correction for radiogenic Sr is negligible. Three distinct types of carbonatite were sampled, and all samples were petrographically assessed to be fresh. Sample PC/3 contains, however, two generations of calcite and some of the calcite may be crust-derived; that would account for the higher $^{87}\text{Sr}/^{86}\text{Sr}$ ratio and this result is rejected.

this case large, and so an approximate measurement of this parameter was made by combining the Pb (~ 25 ng) from two blank runs.

The diagram shows the corrected and uncorrected Pb isotopic ratios in relation to Pb growth curves. In view of the limited nature of the data these results must be treated with caution, but it seems that the μ value for the source region of these diamonds is close to 9.0 and that if a single-stage model is assumed, the age is close to 1,500 Myr. Whether this age refers to the time of pipe formation (assuming that isotopic equilibrium was maintained between Pb in possibly older diamonds and in the surrounding mantle until pipe formation), or to the time of diamond formation itself, is unknown. Further data are needed; a prime objective of such work would be to detect possible mantle depletion in the lithophile elements, as reflected by changing μ values with time.

A $^{87}\text{Sr}/^{86}\text{Sr}$ ratio for carbonatite from Premier, of 0.7028 ± 0.0003 has been reported⁹, and the results of three further measurements are listed in Table 2. The kimberlite found at Premier is considered as too altered to warrant analysis (see ref. 10), but the fresher carbonatites, if derived from an original kimberlite magma rich in carbonates, should have the same $^{87}\text{Sr}/^{86}\text{Sr}$ ratio as the original kimberlite. The fact that the corrected $^{87}\text{Sr}/^{86}\text{Sr}$ ratio for Premier diamonds (0.7028 ± 0.0006) is almost identical with the results listed here for carbonatites from the same source, suggests a genetic relationship between diamonds and the original kimberlite magma. The observed $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is lower than the main trend for basic rocks from southern Africa³; layering of the mantle is implied, with the deep-seated source region of the diamonds characterised by low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios.

We particularly thank Professor W. F. Slawson, who suggested this project, the personnel of de Beers Diamond Sorting Office, Kimberley, for their cooperation; the South African Atomic Energy Board for the use of their mass spectrometer; and I. J. D. van der Westhuizen for assistance with the measurements. Dr R. Danchin provided the carbonatite samples and their petrographic assessment. This work was supported financially by the Anglo-American Corporation.

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Skull of *Palaechthon nacimienti*

SPECIES in or near the ancestry of living primates first appear in the late Cretaceous and early Palaeocene of North America. Subsequent adaptive radiation of the *Purgatorius*-like ancestral stock produced the plesiadapoid families (Plesiadapidae, Carpolestidae, Paromomyidae) of the middle and late Palaeocene. Specialised members of all three families survived into the early Eocene, the paromomyid genus *Phenacolemur* persisting into the late Eocene. Most of the plesiadapoid species are known only from incomplete dentitions. In 1948, a crushed but nearly complete skull of a paromomyid was recovered from strata of middle Palaeocene age in the Kutz Canyon area of the San Juan Basin, New Mexico. The specimen has been described by Wilson and Szalay¹, who assign it to a new species (*P. nacimienti*) of the genus *Palaechthon*, known also from the mid-Palaeocene of Montana and Wyoming. The loss of the upper and lower first premolars excludes *P. nacimienti* from the ancestry of some of the Eocene prosimian lineages. Nevertheless, its persistently primitive molar morphology suggests that it may more closely resemble the last common ancestor of the plesiadapoids and the Eocene primates of modern aspect than do other plesiadapoids for which cranial remains are known. Skulls or partial skulls are known for dentally more specialised genera of each plesiadapoid family: *Plesiadapis* (Plesiadapidae), *Carpolestes* (Carpolestidae), and *Phenacolemur* (Paromomyidae). We present here a reconstruction of the skull of *P. nacimienti*, together with some preliminary functional interpretations of its cranial and dental anatomy.

The skull is unevenly crushed in the dorsoventral plane and lacks the zygomata and the anterior extremity of the rostrum. Its caudal end is missing, including the greater portion of the petrosals and basioccipital, the occipital condyles and foramen magnum, and the corresponding portions of the skull roof. The basicranium is badly fragmented, and foramina cannot be identified with certainty. The ear region is represented only by dubious fragments of the anterior tip of the petrosal; the bulla is completely lost. (The original description of this specimen¹ stated that "... there are signs that a sizable bulla was present," and that "the pterygoid crests are only 0.7 mm apart". The authors may have mistaken fragments of the widely-divergent pterygoid laminae for fragments of the bullae.) Relative brain size cannot be estimated from the preserved material.

Each of us attempted an independent reconstruction of the skull. The results differed in only a few details, which were resolved in preparing Fig. 1. Linear measurements of the skull

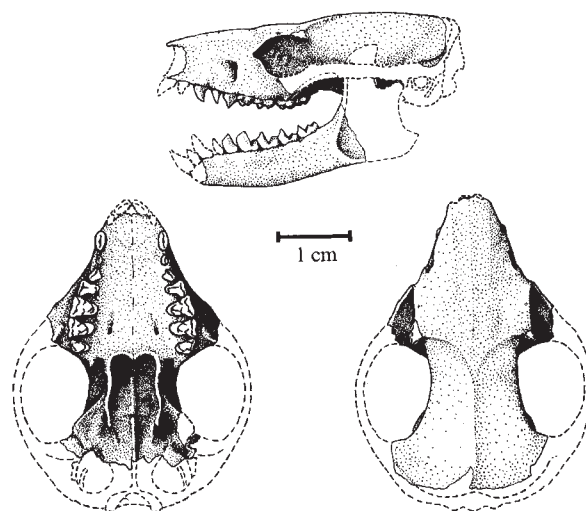


Fig. 1 Ventral, lateral, and dorsal views of the skull of *Palaechthon nacimienti*, based principally on Univ. Kansas Nat. Hist. Mus. No. 9557 and No. 9559. Specimen UKMNH No. 7903, which was originally included in the hypodigm of *P. nacimienti*, represents a new species and accordingly was omitted from consideration here.

and teeth were compared with similar data for extant prosimians, tree shrews, elephant shrews, dermopterans, squirrels, didelphids and phalangerids, as an aid in assessing the adaptive significance of the fossil's morphology.

The cheek teeth of *Palaechthon* show a constellation of features similar to that seen in the extant insectivorous prosimians *Loris*, *Arctocebus*, *Tarsius*, *Galago demidovii* and *Galago senegalensis*. The molars are relatively large and heavily worn. The lower molar crowns of *Palaechthon* are relatively high, and exhibit pronounced concavely-curved leading edges of wear surfaces 1-4. (These and related terms used here are defined in ref. 2.) In mastication, these edges were moved dorsally and anteromedially past matching pairs of reciprocally-curved upper molar blades. The leading edges of upper and lower wear surfaces 5 and 6 are also long. These features are correlated with shearing functions and resistance to wear, and are reduced in more frugivorous extant prosimians³.

In *Palaechthon*, the origin of the anterior temporalis muscle extends forward to a point above the posterior upper molar. The faint temporal lines converge to form a midsagittal line which extends over approximately the posterior 70% of the temporal fossa. The linea obliqua of the mandible, which strengthens the coronoid process against the pull of the temporalis and provides an insertion for the anterior and zygomatic parts of that muscle, is robust and in high relief relative to the masseteric fossa behind it. These features suggest that the principal axis of the temporalis fibres was nearly vertical with respect to the occlusal plane, and situated more anteriorly than is typical of extant prosimians. Similar features are seen among rodents, and are pronounced in a larger and later plesiadapoid from Europe, *Plesiadapis tricuspidens*⁴. The origin of the superficial masseter is marked by a crest on the ventral surface of the orbital margin, above the buccal edge of the second upper molar. This origin is farther forward than in most extant prosimians, and implies a more horizontal orientation for this muscle, as in lorises.

The medial and lateral pterygoid laminae diverge widely, enclosing a large pterygoid fossa. If the origin of the medial pterygoid was restricted to this fossa, as it is in some extant members of the Insectivora, it was probably relatively larger than in extant prosimians of comparable size. If this muscle also had a superficial head arising from the medial orbital wall, as in *Galago*, *Tarsius*, *Tupaia* and *Felis*, it must have been exceptionally large. *Palaechthon*'s well-developed anterior temporalis, enlarged medial pterygoid and horizontally oriented superficial masseter may be in part related to the use of the anterior lower incisors in protrusive prying, shovelling or other ingestive movements.

The orbital margins are deformed but largely preserved. The supraorbital shelf is well developed, and a short superior postorbital process is present. The shape of the superior orbital margin indicates that a complete postorbital bar was probably absent. The dihedral angle between orbital and midsagittal planes (measured from a sculpted reconstruction) was about 35°, and the line of intersection of these two planes must have been approximately parallel to the skull roof. In these respects, *Palaechthon* resembles terrestrial sciurids^{4,5}. Visual-field overlap could not have been extensive.

The orbital diameter of *Palaechthon* was approximately 7 mm. This is smaller than would be expected for extant prosimians, tree shrews, elephant shrews, sciurids, dermopterans or arboreal didelphids of comparable body size. Nocturnal terrestrial foragers like *Echinosorex* and *Monodelphis* resemble *Palaechthon* in relative orbital diameter.

The interorbital breadth of *Palaechthon* is unusually great. In extant mammals, three principal factors influence this dimension. First, in animals with relatively gracile zygomata, orbital convergence necessitates reduction of interorbital breadth⁶. Second, interorbital breadth may be increased for the transmission of exceptional compressive stresses generated in mastication or incisal biting, as in *Daubentonina*, *Dactylopsila*⁷,

*Alouatta*⁸ and colobines. Third, interorbital breadth may increase in conjunction with an enlargement of the olfactory fossa, as in *Nasua*. The last two of these factors probably operated in *Palaechthon*; the preserved parts of the skull demonstrate the resulting lateral position of the orbits (accentuated by the supraorbital shelf) and the large olfactory fossa.

Compared with extant prosimians and tupaiine tree shrews, *Palaechthon* has a large infraorbital foramen, implying that the innervation and blood supply of the rhinarium and vibrissal roots were correspondingly well developed. The relative size of this foramen resembles that of living hedgehogs and carnivores.

In summary, the known cranial and dental remains of *P. nacimienti* imply that it relied more on tactile and olfactory information than on vision in its foraging activities. The well-developed anterior temporalis suggests that powerful occlusal forces could be exerted in mastication. The molar morphology indicates a predominantly insectivorous diet. An adaptive mode involving substantial amounts of nocturnal foraging on the forest floor is consonant with the observed morphology. Squirrel-like arboreal habits involving diurnal foraging for plant foods, or visually-directed predatory habits like those of many small marsupials or extant insectivorous prosimians, seem less likely.

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ADP binding to relaxed scallop myofibrils

CALCIUM regulates muscle contraction in all species which have been studied. Muscle relaxes in the absence of calcium and contracts when it is present in micromolar concentrations¹⁻³. Contraction is caused by the interaction of myosin with ATP and with the actin of the thin filament, and the effect of calcium is to modulate this interaction¹⁻⁸. Although these features apply to all muscles, the location of the calcium binding and thus the mechanism of its action differs in different phyla; regulation may be associated with the myosin, or the thin filaments or both^{2,3,7}.

The mechanism of the myosin ATPase in vertebrate muscle, in which regulation is thin filament-linked, has been widely studied. Myosin rapidly binds and hydrolyses ATP to form a myosin-ADP-P_i intermediate^{9,10}. The breakdown of this complex is the rate limiting step of hydrolysis

and actin accelerates this rate¹¹. Therefore, in relaxed vertebrate muscle where the actin-myosin-ADP-P_i interaction is blocked by the troponin-tropomyosin of the thin filaments, ADP and P_i are found bound to myosin in the steady state¹².

In molluscs, on the other hand, calcium regulation is myosin-linked and it is not known whether, in the absence of calcium, the hydrolysis of ATP, itself, is blocked and myosin-ADP-P_i is not formed, or whether myosin-ADP-P_i is formed but its association with the thin filaments is blocked. In this study we show that in molluscs, as in vertebrates, ADP is bound to myosin in relaxing conditions.

Myofibrils were prepared from both fresh and glycerinated *Aequipecten irradians* striated adductor muscles. Muscles were glycerinated according to Kendrick-Jones *et al.*⁷ in a solution consisting of 50 mM NaCl, 1 mM MgCl₂, 5 mM phosphate buffer (pH 7.0), 0.1 mM sodium azide and 50% glycerol, and stored at -20°C for one month. To prepare myofibrils, muscles were homogenised in a Sorvall Omni mixer in 5 mM piperazine-N,N'-bis-2-ethanesulphonic acid (PIPES), pH 7.1, 5 mM ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), pH 7.0, 50 mM KCl and 5 mM MgCl₂, and sedimented at 12,000g for 10 min. The pellet was rehomogenised in the same solution and resedimented at 500g for 10 min twice. A homogenous suspension of myofibrils, containing 15–20 mg protein ml⁻¹, was embedded in a 1% agarose gel as previously described^{12,13}. Blocks of the gel (100 to 200 nl) were cut out and incubated at 2°C in relaxing solution containing ¹⁴C-ATP at 200 mCi mmol⁻¹; ¹⁴C-mannitol was used as a volume

of the data¹⁴ showed a maximum ADP binding of 2.69 ± 0.15 nmol per mg protein, and the dissociation constant was 9 mM ± 2 s.e. The amount of ADP bound was almost identical to the estimated quantity of myosin sites in the muscle (2.75 nmol mg⁻¹ based on a 62% myosin content⁸ and a weight of 225,000 daltons per active site^{15,16}). Myofibrils from fresh muscle were about 90% calcium sensitive, bound slightly less ADP (2.45 ± 0.15 nmol mg⁻¹) and had a slightly higher dissociation constant (12 ± 3 mM). The smooth ('catch') muscle of *Aequipecten*, which has a smaller myosin content than the striated muscle, also bound only ADP in relaxing conditions, with a maximum binding of 0.75 nmol per mg protein.

These results show that the steady-state intermediate of relaxed myosin ATPase of scallop muscle is M·ADP (presumably the P_i moiety of ATP is also bound in this complex^{9,10,12}); thus, the decomposition of this complex is rate limiting. It follows that calcium activation cannot act at the hydrolytic step: M·ATP ⇌ M·ADP·P_i, as this reaction is not rate limiting, but must affect the subsequent reaction of M·ADP·P_i with actin. It was previously shown⁸ that scallop myosin has a reduced apparent affinity for actin in relaxing conditions; in this paper the cause of this has been explained. From the results of nucleotide binding experiments on vertebrate, insect, and molluscan muscle, it seems likely that calcium regulates the actomyosin ATPase cycle at the same step in each, even though the control site may be located on myosin as in scallop⁷, on the thin filaments as in vertebrates^{1,2,11,12}, or on both the myosin and the thin filaments as in insects, such as *Lethocerus*^{3,12,17}.

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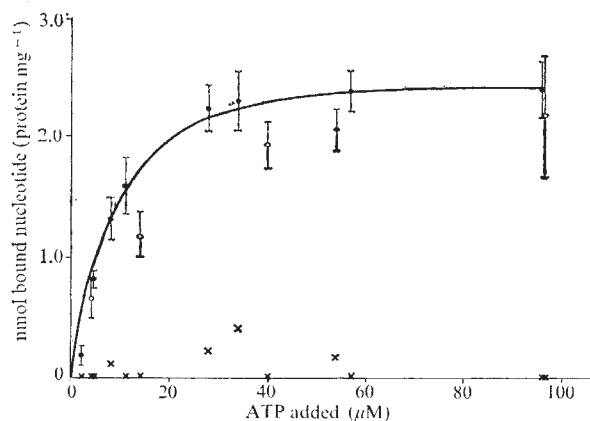


Fig. 1 Nucleotides bound by scallop striated muscle in relaxing conditions in the presence of different concentrations of MgATP. Conditions: 5 mM PIPES buffer; 5 mM EGTA; 5 mM MgCl₂; 50 mM KCl; 4 mM creatine phosphate; phosphocreatine kinase (5 mg ml⁻¹); 1 mM sodium azide 0–100 μM ¹⁴C-ATP, ¹⁴C-mannitol, pH 7.1, *i* = 0.095, 2°C. ADP bound to glycerinated, ●, and fresh, ○, myofibrils; ×, corresponding ATP binding; vertical bars, ± 5% fiducial limits of eight assays.

marker and an ATP regenerating system was included in the medium (Fig. 1). After 3 min, the gel blocks were transferred to trichloroacetic acid and the bound nucleotide content was determined. The details of this procedure have been described in a previous paper^{12,13}. The myofibrillar ATPase (that is, Mg-ATPase) was measured in a pH stat as previously described at 2°C and 25°C.

In relaxing conditions (ATP, no calcium) where the Mg-ATPase was at least 97% inhibited, myofibrils from glycerinated scallop striated muscle bound a large quantity of ADP and only minimal amounts of ATP and AMP (Fig. 1). Assuming a simple binding, a nonlinear regression analysis

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In vitro activity of cells from genetically lethal embryos of *Drosophila*

MUTATIONS that affect particular embryological events can help to elucidate the developmental interactions and cellular and biochemical mechanisms. In *Drosophila* much of the work^{1,2} in developmental genetics has been done on strains that carry chromosomal aberrations, particularly deficiencies; or on strains that segregate aneuploid progeny, such as X-linked stocks.

Deep orange (*dor*) is an X-linked (1–0.3) recessive mutant of *D. melanogaster*³. In addition to an alteration in pteridine pigments of the eye⁴, *dor* causes a peculiar type of sterility³⁻⁶. When *dor* females are mated to *dor* males, all offspring die during the course of embryonic development. When heterozygous females are mated to *dor* males, however, all of the expected progeny types survive and develop normally. This indicates that the viability of *dor* embryos depends on the maternal as well as the zygote genotype.

Thus *dor* embryos survive only if an essential developmental component is provided by the egg cytoplasm produced by heterozygous mothers. This repair of *dor* egg defect by the essential component has been recently confirmed by an injection experiment in which cytoplasm from unfertilised normal eggs was injected into *dor* embryos at the syncytial preblastoderm stage of development⁷. The present experiment reveals that some types of cells from *dor* embryos proliferate actively and manifest their function for prolonged periods when they are cultured in a chemically defined medium with some macromolecular supplementations, especially when the wild-type egg extract was added to culture medium.

The *dor* strain used was a balanced stock carrying *CIB* X chromosome. Eggs collected from matings of *dor/dor* females and *dor/Y* males were dechorionated by a 6-min treatment with 3% sodium hypochloride. The eggs were then rinsed with distilled water and transferred to physiological salt solution, in which they were allowed to develop at 25° C. The developmental stages of dechorionated eggs were readily determined through the transparent vitelline membrane under a binocular microscope. Among 463 eggs obtained from the above matings, 19 (4.1%) died before gastrulation, 179 (38.7%) died by abnormal gastrulation, 204 (44.0%) died after the stage of sac-like midgut, 61 (13.2%) died after muscular movement and none hatched.

Dor embryos which developed beyond gastrulation were sterilised in 70% ethyl alcohol for 10 min, rinsed three times with sterile salt solution, and used for *in vitro* cultivation. The procedure for cultivation of *Drosophila* embryonic cells has been previously described⁸. Embryos were torn into small fragments with a pair of fine needles and cultured in T-5 flasks in medium K-17 (ref. 9) supplemented with 0.1 mg ml⁻¹ fetuin and 15% foetal bovine serum.

After several hours of cultivation, spindle-shaped muscle cells came out from the cut ends of tissue fragments (Fig. 1a).

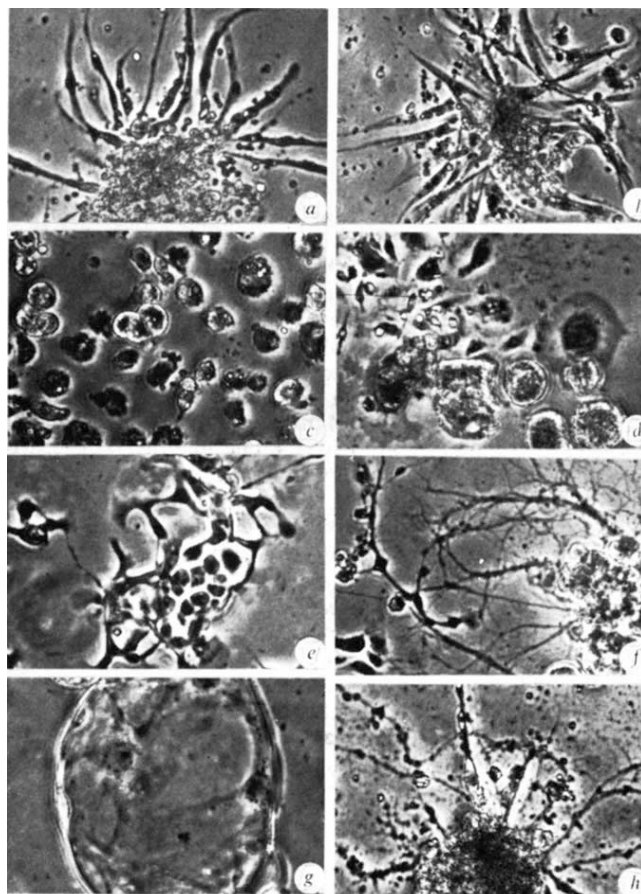


Fig. 1 Various types of cells from *dor* lethal embryos of *Drosophila melanogaster* ($\times 450$). *a*, Muscle cells after 4-d cultivation; *b*, muscle cells pulsating synchronously after 34-d cultivation; *c*, epithelial cells with many cytoplasmic granules after 3-d cultivation; *d*, epithelial cells showing chitinous pigmentation after 7-d cultivation; *e*, fibroblastic cells after 3-d cultivation; *f*, nerve cells with network of nerve fibres after 8-d cultivation; *g*, a cellular sphere after 10-d cultivation with wild-type egg extract; *h*, nerve fibres with deposits of small droplets on them after 10-d cultivation with wild-type egg extract.

They were 50–100 μ m in length and had an ovoid nucleus. They gradually increased in number around the original tissue fragments. The formation of syncytial complexes, which has been observed in cultures of wild-type embryonic cells, was not found in cultures of *dor* embryonic cells. Some of these muscle cells pulsated at a particular interval for each cell. When they were in contact with each other, they pulsated synchronously. This pulsation continued for more than 5 weeks under culture conditions employed (Fig. 1b).

Flat polygonal epithelial cells were also found at the initial

Table 1 Growth and differentiation of various types of embryonic cells from *Drosophila melanogaster in vitro*

Cell type	Growth and differentiation	Oregon-R [*]	Embryonic cells from <i>dor</i> without OEE*	<i>dor</i> with OEE
Muscle cells	Pulsation	+	+	+
	Syncytium formation	+	—	±
Epithelial cells	Monolayer sheet	+	±	+
	Maturation	+	+	+
Fibroblastic cells	Monolayer sheet	+	+	+
Cellular spheres	Sphere formation	+	—	+
Small cells	Monolayer sheet	+	—	—
	Regular arrangement	+	—	—
Nerve cells	Fibre extension	+	+	+
	Fibre branching	+	+	+
	Droplet formation	+	—	+

* Oregon-R egg extract.

stage of cultivation. They grew slowly and increased gradually in size with many cytoplasmic granules (Fig. 1c). It seemed that their maturation proceeded. After cultivation for 7 d a chitinous pigmentation (coloured brown) was observed in the cytoplasm of some cells (Fig. 1d).

Fibroblastic cells which were most motile appeared after 3-d cultivation (Fig. 1e). They migrated rapidly by extension and contraction of their cytoplasmic processes. In cultures of wild-type embryonic cells it has been found that fibroblastic cells formed balloon-like cellular spheres⁸. In the present cultures of *dor* embryonic cells, however, no such cellular spheres were observed.

Some nerve fibres extended from nerve cells. They increased in length and formed many branches. After cultivation for 8 d nerve fibres made contact with each other and formed a nerve network (Fig. 1f). In further cultivation, however, the deposition of small droplets, which has been found in cultures of wild-type embryonic cells and seems to be some secretion from the nerve fibres, were not observed in cultures of *dor* embryonic cells.

In cultures of wild-type embryonic cells, some groups of extremely small cells which were epithelial in morphology, about 3 μm in diameter and seemed to be imaginal disk cells has been observed⁸. In the cultures of *dor* embryonic cells, no such small cells were detected in identical culture conditions.

The characterisation and phenotypic expression of cell types appearing in cultures of *dor* embryonic cells are summarised in Table 1. In this table, the findings in cultures of wild-type embryonic cells are also listed for comparison. This indicates that mesodermal cells such as muscle cells and fibroblastic cells from *dor* embryos were maintained in a functionally active state for a relatively extended period over the prospective lethal phase of the *dor* embryos, although some defects in properties such as syncytium formation of muscle cells were found. On the other hand, ectodermal cells such as epithelial cells, small cells, and nerve cells grew scarcely and manifested only a part of their respective behaviour and functions in the *in vitro* conditions employed.

Unfertilised eggs collected from the wild-type strain, Oregon-R, were homogenised in culture medium and centrifuged at 2,000 r.p.m. for 15 min. When *dor* embryonic cells were cultured in medium containing the wild-type egg extract (100 eggs ml^{-1}) obtained by this method, defects in behaviour and functions of ectodermal cells were found to be almost repaired (Table 1); syncytium formation of muscle cells, formation of cellular spheres (Fig. 1g), and droplet formation on nerve fibres (Fig. 1h). Even in cultures with the wild-type egg extract, the growth and formation of regular arrangement of small cells were still not observed.

Since the *dor* mutant is known to be abnormal in pteridine metabolism, some pteridines may have an effect on the normal growth and differentiation of *dor* embryonic cells. The procedure for *in vitro* cultivation of *dor* embryonic cells seems to offer an experimental approach to analysing the effective substances which are produced by the normal allele of *dor* gene and may be necessary for normal development.

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Is calcium ionophore a universal activator for unfertilised eggs?

WE have previously exposed sea urchin eggs to micromolar amounts of the divalent transporting ionophore A23187, and observed every parameter of normal fertilisation. The cortical reaction with elevation of the fertilisation membrane, the plasma membrane conductance changes, the respiratory burst, and increases in protein and DNA synthesis were all initiated in the usual fashion¹. These activations of *Lytechinus pictus* and *Strongylocentrotus purpuratus* eggs were independent of the ionic composition of the external solutions¹. A23187 seemed to act by releasing intracellular Ca^{2+} , for eggs pre-loaded with ^{45}Ca showed a twentyfold increase in ^{45}Ca -efflux when activated by ionophore or fertilisation¹. Measurements of free and bound calcium and magnesium in homogenates of unfertilised eggs showed that most of the Mg^{2+} was already available in soluble form, whereas the Ca^{2+} was sequestered but available for release¹. After we found that 50 μM A23187 could activate eggs of the mollusc *Acmaea insessa* and the protochordate *Ciona intestinalis* to undergo several abnormal cleavages, we wondered whether ionophore activation might be a general phenomenon. To our knowledge artificial parthenogenesis has not been obtained with *Acmaea* and *Ciona* eggs by other methods. We have now found that calcium ionophore activation is common to several species widely separated phylogenetically.

We used three species whose egg activation has been well described in the literature; the bat-star, *Patiria miniata* (Asteroidea), the toad, *Xenopus laevis* (Anura), and the hamster, *Mesocricetus auratus* (Mammalia). A 5 mM stock solution of A23187 (obtained from R. L. Hamill, Eli Lilly Co., Indianapolis) was prepared in dimethylsulphoxide (DMSO) and stored in the dark. Appropriate controls were run with DMSO and indicated no effect. When the stock ionophore solution was diluted for use with oocytes, the solutions were stirred continuously to ensure good mixing, since the ionophore is very insoluble in water and there is some precipitation. Final concentrations given here are therefore maximum estimates.

Isolated ovaries of *P. miniata* were teased to free the oocytes, which were stored as premeiotic cells in filtered seawater at 16°C. When oocytes were first incubated in the hormone 1-methyladenine (1-MA) $1 \times 10^{-5}\text{M}$ for 30 min to induce maturation²⁻⁴, exposure to ionophore (5–25 μM) initiated a cortical reaction indistinguishable from that initiated by sperm.

Meiosis continued in these activated eggs and two polar bodies were produced as in eggs treated with 1-MA only. Usually, the activated eggs did not divide. After 20 h more than 90% were still one cell. One two-cell and one 12–16-cell stages were seen.

Ionophore activation did not depend on exogenous calcium. Eggs pretreated with 1-MA could be activated by 5–25 μM ionophore in Ca^{2+} -free seawater (Na^{+} substituted for Ca^{2+} plus 2 mM ethylene glycol bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA)).

X. laevis females were treated as described by Wolf and Hedrick⁵, to obtain eggs, which were expressed into De Boer's solution (0.11 M NaCl, 0.0013 M KCl, 0.00440 M CaCl_2 plus solid NaHCO_3 to pH 7.2) and 5–10 oocytes per ml were placed into small syracuse dishes. Criteria of activation

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Table 1 Activation of *X. laevis* eggs by A23187

Solution	No. activated	No. treated	% activated*
5 μ M A23187 in 0.1% DMSO-De Boer's	13	15	87
10 μ M A23187 in 0.2% DMSO-De Boer's	21	25	84
De Boer's	0	5	0
0.1% DMSO-De Boer's	0	18	0
0.2% DMSO-De Boer's	0	25	0
5 μ M A23187 + Ca^{2+} -free De Boer's + 2 mM EDTA	10	10	100
Ca^{2+} -free De Boer's + 0.1% DMSO	0	6	0

*Percentage observed with both cortical reaction and pigment migration.

were first, elevation of a fertilisation membrane and, second, contraction or capping of pigment from vegetal to animal hemisphere.

As Table 1 shows, 5–10 μ M A23187 elicited both cortical reactions and pigment migration within 2–3 min: both changes were complete by 5–13 min. These times are similar to those observed after normal fertilisation. The same activations were observed in Ca^{2+} -free De Boer's medium supplemented with 2 mM ethylene-diaminetetraacetic acid (EDTA).

Mature unfertilised eggs of *M. auratus* were obtained from oviducts of unbred females 15–17 h after injection of human chorionic gonadotrophin⁶. The eggs were freed from surrounding cumulus cells by treatment for 15 min (25°C) with 0.1% bovine testicular hyaluronidase (300 USP U ml⁻¹; Nutritional Biochemicals) in Biggers, Whitten and Whittingham (BWW) medium and rinsed thoroughly with Ca^{2+} - Mg^{2+} -free BWW. (The medium was modified by replacing albumin with 0.2% polyvinylpyrrolidone. pH was adjusted to 7.3 by addition of a small amount of N/5 HCl.)

The eggs were placed in 0.2 ml of either normal or Ca^{2+} - Mg^{2+} -free BWW (pH 7.3) containing freshly diluted 3–20 μ M A23187 and incubated under mineral oil (Squibb) in a watch-glass in an air atmosphere at 37°–38°C. At various times the eggs were examined with a phase contrast microscope to determine the condition of cortical granules and female pronucleus.

Continuous exposure to high concentrations (10–20 μ M) of A23187 was detrimental to the eggs, resulting in disintegration of the cytoplasm. Exposure to a lower concentration (3 μ M), activated the eggs, the extent of activation being greater in Ca^{2+} - Mg^{2+} -free media (Table 2a) than in the complete medium (Table 2b). The rate of cortical granule breakdown and pronuclear development in Ca^{2+} - Mg^{2+} -free media was comparable with the rate in sperm-activated eggs⁸. The extent of cortical granule breakdown, however, was usually less in A23187-activated eggs than in those activated by sperm. Qualitative estimates showed 80–90% breakdown of granules in eggs activated in Ca^{2+} - Mg^{2+} -free media and a less complete breakdown in eggs activated in the complete medium. Activation of nuclear events were also slower in the complete medium. These differences may have resulted from decreased solubility of the ionophore in the presence of divalent cations.

Brief exposure to A23187 induced activation of the eggs. For example, when the eggs were exposed to 3–10 μ M A23187 in Ca^{2+} - Mg^{2+} -free BWW for 2 min then washed and left in Ca^{2+} - Mg^{2+} -free BWW, they all showed breakdown of the cortical granules within 30 min and well developed female pronuclei were formed in 2 h.

The activation by A23187 induced a zona reaction identical to that resulting from normal fertilisation. This is a change in the zona pellucida surrounding the egg, which prevents sperm penetration^{9,10}. Unfertilised cumulus-free eggs were rinsed with Ca^{2+} - Mg^{2+} -free BWW washed and left in Ca^{2+} - Mg^{2+} -free BWW, incubated for 2 min in 3–10 μ M A23187 in Ca^{2+} - Mg^{2+} -free BWW for 30 min at 37°–38°C. The eggs were then transferred to a medium containing capacitated hamster sperm. The spermatozoa were incubated in a mixture of heat-inactivated rabbit serum and Tyrode solution (1:1) for 3–4 h to induce capacitation and the acrosome reaction before mixing with the eggs. The eggs were incubated with capacitated sperm at 37°–38°C for 2 h. No spermatozoa penetrated the zona pellucidae of these eggs (45 eggs tested). Control eggs (38 tested) treated in the same way except for exposure to A23187 were all penetrated by spermatozoa.

Calcium has long been implicated to some extent in the activation of marine eggs. The usual procedure to induce some degree of parthenogenetic development was to substitute high concentrations of calcium for sodium in artificial seawaters^{11,12}. Also consistent with a Ca^{2+} involvement was the observation that an increase in free cytosol calcium occurs in eggs which have just been fertilised^{13,14}. Previous results with the sea urchin indicate that activation can result from the release of intracellular stores of calcium induced by a calcium ionophore¹. The Ca^{2+} -ionophore can also activate a wide

Table 2 Activation of hamster eggs by A23187 (continuous exposure)

(a) 3 μ M A23187 in Ca^{2+} - Mg^{2+} -free BWW							
% of egg nuclei exhibiting*:							
Time examined (min)	Cortical granules	Metaphase-II	Late anaphase-II to Early telophase-II	Mid-telophase-II	Early pronucleus	Well developed pronucleus	% of eggs degenerated
0	++++	100	0	0	0	0	0
30	+	0	100	0	0	0	0
40	+	0	0	100	0	0	0
60	+	0	0	0	100	0	0
80	+	0	0	0	14.3	78.5	7.2
120	+	0	0	0	0	73.5	26.5
(120 min control) no A23187 treatment	++++	100	0	0	0	0	0
(b) 3 μ M A23187 in normal (Ca^{2+} - Mg^{2+} -containing) BWW							
0	++++	100	0	0	0	0	0
30	++++	100	0	0	0	0	0
40	+++ to ++++	80	20	0	0	0	0
60	++ to ++++	53.8	23.2	23.0	0	0	0
120	++ to ++++	21.4	0	0	21.4	42.9	14.3

* 30–40 eggs in each group.

variety of species of eggs as reported here; and these activations of marine, amphibian and mammalian oocytes are also independent of external calcium. We now propose that release of intracellular ionic calcium may be the universal factor promoting activation of egg metabolism at fertilisation.

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Concanavalin A-induced agglutination of *Acanthamoeba*

VIRULENT strains of *Acanthamoeba* produce cytopathic effects in mammalian tissue cultures¹⁻³, and cause acute meningoencephalitis in laboratory animals after intranasal inoculation⁴. In humans, *Acanthamoeba* seem to produce cerebral lesions in defence-weakened individuals⁴⁻⁷. We have begun studies of the cell surface of *Acanthamoeba* since virulent strains must make direct contact with a host cell before initiation of degenerative changes in mammalian tissue cultures^{2,3}. The sensitivity of avirulent and virulent strains to concanavalin A (con A)-induced agglutination was investigated first as there was no evidence that carbohydrate-containing components were exposed at the cell surface of *Acanthamoeba*. Our results indicate that both avirulent and virulent *Acanthamoeba* were sensitive to con A-induced agglutination. In contrast to the results of studies of avirulent and virulent *Entamoeba histolytica*⁸, however, the avirulent strain was more sensitive to con A.

The avirulent Neff strain⁹ of *A. castellanii* and a virulent A-3 strain of *A. culbertsoni*¹⁰ were cultured under constant axenic, suspension growth conditions as described previously¹¹. The strains were removed from exponential cul-

tures of approximately the same age, concentrated by centrifugation and washed three times with calcium-magnesium-free-phosphate-buffered saline (CMF-PBS). Cells (5×10^5 ml⁻¹) were placed in Microtest tissue plates (Falcon) and, unless otherwise indicated, treated with the specified concentration of con A (crystallised three times; Miles-Yeda) with constant agitation at 22° C for 5 min. Agglutination was scored immediately on a scale of 0 to 4+ under an inverting microscope. Studies with ferritin-labelled con A (prepared as before¹²) were performed either at 4° C or 22° C, using solutions pre-equilibrated before addition of the cells. After incubation for 30 min, cells were maintained at 4° C or 22° C during three washes with CMF-PBS and preparation for electron microscopy.

Table 1 shows that under identical conditions, the avirulent strain of *Acanthamoeba* consistently exhibited twice the sensitivity to con A-induced agglutination than that shown by the virulent strain. Simultaneous addition of 3.5 mM α -methyl-D-mannopyranoside abolished the agglutination of both strains. Nonspecific sugars such as lactose affected agglutination only at significantly higher concentrations (Table 1).

We further investigated whether agglutination could be affected by various treatments. Agglutination of both strains, tested at different concentrations of con A (avirulent: 0.5, 5 or 25 μ g ml⁻¹; virulent: 5, 25, or 50 μ g ml⁻¹), was unaffected after pretreatment for 15 min at 31° C with trypsin or pronase (0.0001 to 0.01 mg ml⁻¹), neuraminidase (*Vibrio cholera*; 15 U ml⁻¹; General Biochemicals) or ethylenediaminetetraacetic acid (0.1–1.0 mM). On the other hand, both strains pretreated for 15 min at 31° C with sodium cyanide (16 mM), sodium arsenite (5 mM) or glutaraldehyde (0.25%) showed no agglutination after exposure to the above concentrations of con A. These results suggested that con A-mediated agglutination of *Acanthamoeba* might depend on free mobility of receptor sites in the membrane and also require ATP. Glutaraldehyde inhibition of con A-induced agglutination of transformed 3T3 fibroblasts has been explained similarly^{13,14}; however, ATP apparently is not necessary for con A-induced agglutination of these cells¹⁴.

As Fig. 1 shows, agglutination of both strains also was inhibited significantly at temperatures of 15° C or less. The temperature sensitivity further appeared to depend on the con A concentrations, and cells treated with con A at 5° C agglutinated rapidly when the temperature was raised to 22° C. Although inhibition of *Acanthamoeba* agglutination at low temperatures might be due to an alteration in the fluidity of the membrane¹⁴, we cannot yet exclude the possibility that the inhibition is due to some other cellular alteration, for example, inhibition of an essential enzyme(s).

Since con A has been shown to bind at low tempera-

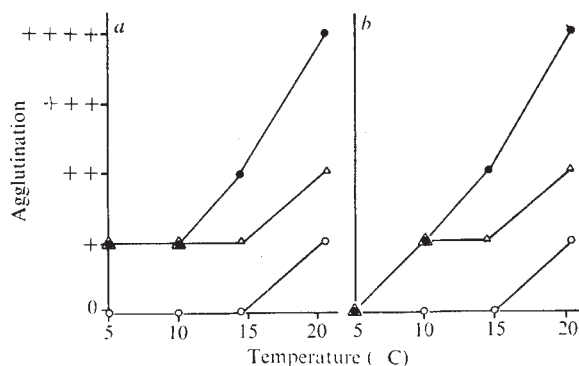


Fig. 1 Effect of temperature on con A-induced agglutination of avirulent (a) and virulent (b) *Acanthamoeba*. Concentrations of con A (μ g ml⁻¹) were (a): ●, 20; △, 10; ○, 1.5; (b): ●, 50; △, 25; ○, 7.5.

Table 1 Con A-induced agglutination of avirulent and virulent strains of *Acanthamoeba*

Addition	Agglutination†	
	Avirulent	Virulent
CMF-PBS*	0	0
Con A‡ (µg ml ⁻¹)		
2.5	+	0
5.0	++	0
7.5	—	+
10.0	+++	—
15.0	++++	++
25.0	++++	++
50.0	++++	++++
Con A§ and α-methyl-D-mannopyranoside (3.5 mM)	0	0
Con A§ and lactose (mM)		
5.0	++++	++++
10.0	++++	++++
25.0	++++	++++
50.0	++++	++++
100.0	+	+

* Cells were placed at a concentration of 5×10^5 cells ml⁻¹ in CMF-PBS and served as controls in all experiments.

† 0, occasional clumps of three or less cells; 2+, ~50% of cells in clumps of 8 to 10 cells; 4+, >95% of cells in large clumps; —, not tested.

‡ Con A was prepared in CMF-PBS.

§ The con A concentration in the competition experiments was 15 µg ml⁻¹ or 50 µg ml⁻¹ for the avirulent or virulent strains, respectively.

tures^{14,15}, *Acanthamoeba* was treated with ferritin-labelled con A at 4° C or 22° C in an attempt to explain the greater agglutination of avirulent compared with virulent cells. In accordance with explanations for the similar sensitivity of transformed or trypsin-treated 3T3 fibroblasts compared with the normal fibroblast, the difference might be due to the amount of con A bound^{14,16}, topographical distribution of the receptor sites¹⁷, and/or mobility of the receptor sites in the lateral plane of the membrane^{23,14}.

Figure 2 shows high magnification micrographs of the cell surfaces of avirulent and virulent *Acanthamoeba* after exposure to ferritin conjugate at 4° C. The conjugate was

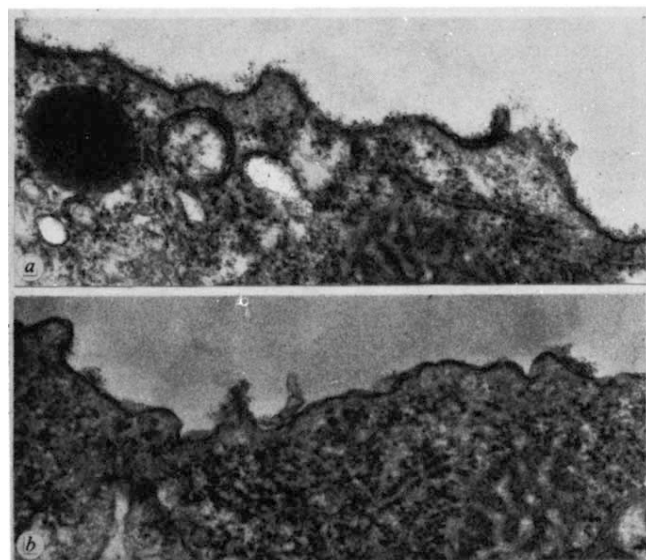


Fig. 2 Electron micrographs of thin sections of avirulent (a) or virulent (b) *Acanthamoeba* exposed to ferritin-labelled con A (80 µg ml⁻¹) at 4° C. Cells were placed at 5×10^5 cells ml⁻¹ in pre-equilibrated solutions of ferritin conjugate and incubated for 30 min. After several washes in CMF-PBS, cells were fixed with glutaraldehyde and osmium tetroxide, embedded in Araldite, and the sections stained with uranyl acetate and lead citrate (a, $\times 36,480$; b, $\times 43,920$).

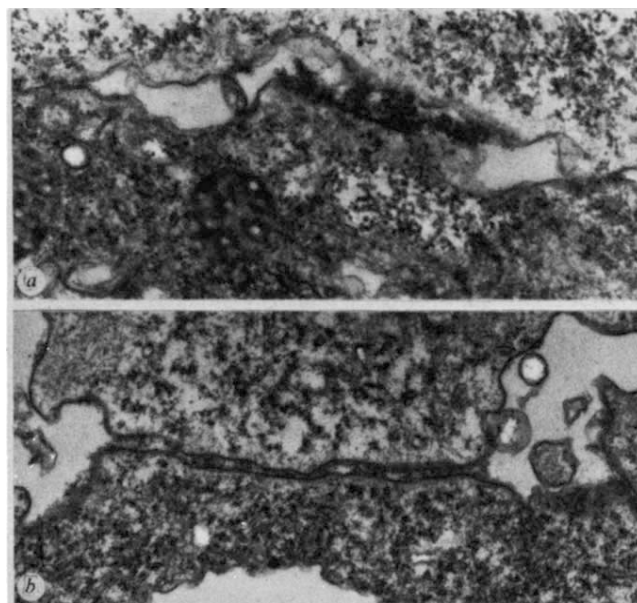


Fig. 3 Electron micrographs of thin sections of avirulent (a) or virulent (b) *Acanthamoeba* exposed to ferritin-labelled con A (40 µg ml⁻¹) at 22° C. After incubation in the conjugate for 30 min, samples of cells were processed as described in Fig. 2. The micrograph shown in (b) was typical of the contact regions of both agglutinated virulent and avirulent amoebae. The micrograph in (a) illustrates the electron-dense, amorphous material with associated ferritin-labelled con A which was found occasionally in contact regions of agglutinated avirulent amoebae (a, $\times 29,280$; b, $\times 19,200$).

distributed randomly with only an occasional cluster of the ferritin label at the surface of the avirulent amoebae (Fig. 2a). In contrast, the surface of the virulent cells appeared almost devoid of associated ferritin-labelled con A. High magnification revealed, however, that the conjugate was localised in discrete clusters on the virulent amoebae surface (Fig. 2b). In both avirulent and virulent cells, little or no label was found within the cytoplasm but, when present, it was enclosed in small membrane-bound vacuoles (Fig. 2a). Sections of control preparations, in which cells were exposed only to ferritin, showed no nonspecific binding of ferritin molecules.

Figure 3 shows electron micrographs of avirulent and virulent *Acanthamoeba* exposed to the ferritin-labelled con A at 22° C and illustrates regions of contact between agglutinated cells in the samples. High concentrations of the conjugate were observed routinely on membrane areas immediately adjacent to and at contact points of both agglutinated avirulent and virulent amoebae. Areas more distal to the contact regions were relatively free of conjugate. Figure 3b, although showing agglutinated virulent amoebae, is typical of the appearance of most contact regions between avirulent cells. Additionally, however, contact regions of avirulent amoebae occasionally contained large amounts of electron dense, amorphous material with associated ferritin-labelled con A (Fig. 3a). Although not illustrated, the ferritin conjugate was clustered on the surfaces of non-agglutinated, single cells in samples of both avirulent and virulent cells treated with the conjugate at 22° C.

In view of the uniform distribution of ferritin conjugate at the surface of avirulent amoebae exposed at 4° C, the greater agglutination of this strain cannot be attributed to the presence of pre-clustered con A receptor sites, which may account for the greater sensitivity of the transformed 3T3 fibroblast to con A¹⁷. Instead, the greater sensitivity of

the avirulent strain probably results because more con A receptor sites (mannose-like residues) are exposed at the surface than on the virulent strain. A greater mobility of the con A receptor sites in the membrane of the avirulent strain may contribute partially to the greater agglutination; however, since the virulent strain appeared capable of clustering con A receptor sites, as evidenced by the large aggregates associated with contact points between agglutinated cells in samples exposed to ferritin conjugate at 22°C, the difference in the membrane fluidity of the strains may be minimal.

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Dissociation of increased hexose transport from initiation of fibroblast proliferation

ALTERATIONS in the permeability of the surface membrane might play a key role in the control of DNA synthesis and cell division¹⁻³. Evidence that changes in hexose transport might participate in growth control comes from reports that uptake of certain hexoses (1) increases after transformation by tumour viruses⁴⁻⁶, and correlates with the expression of the transformed phenotype⁷; (2) decreases when untransformed fibroblasts reach a density-inhibited monolayer^{8,9}, and (3) rapidly increases after initiating cell division with serum or other agents^{8,9}. We now report, however, that increased hexose transport and initiation of proliferation can be uncoupled in density-inhibited 3T3 mouse fibroblasts. Levels of cortisol which initiate DNA synthesis and division of these cells^{10,11} actually decrease hexose transport. In addition, some serum components increase hexose transport without initiating proliferation.

Mycoplasma-free 3T3 Swiss mouse fibroblasts (clone 42) were grown as previously described¹². Medium components were purchased from Gibco. Density-inhibited cultures were prepared by plating 30 or 50 mm Nunc plastic tissue culture dishes with 1.7×10^4 cells cm^{-2} in 2 ml (for 30

Table 1 Stimulation of DNA synthesis and cell division by cortisol and serum

Addition	cpm ³ H-thymidine	cells $\times 10^{-4}$
	10 ⁵ cells	$\frac{\text{cells}}{\text{cm}^2}$
None	235	3.6
3×10^{-8} M Cortisol	1,267	4.9
2.5% Serum	889	4.0
5.0% Serum	1,372	4.5
7.0% Serum	1,622	5.2

Cells were grown to confluency and cortisol or fresh serum was added to cultures. DNA synthesis and cell number were determined at 24 and 72 h respectively after these additions as previously described¹⁰.

mm dishes) or 4.5 ml (for 50 mm dishes) of culture medium containing 10% calf serum. The cells grew to a final saturation density of 3.5×10^4 to 4.2×10^4 cells cm^{-2} over 3 d. Hexose uptake was measured using the non-metabolisable hexose analogues 3-O-methyl-D-glucose and 2-deoxy-D-glucose. Both of these sugars are transported into cells by facilitated diffusion³. Kinetic studies have indicated that they are transported by the same system that transports glucose.

To determine the extent of correlation of initiation of proliferation and increased hexose transport, we added fresh serum (5.0%) or the glucocorticoid steroid hormone, cortisol (3.0×10^{-6} M) and measured DNA synthesis, cell number and uptake of hexose analogues. These additions caused similar increases in the rate of DNA synthesis at 24 h and cell number at 72 h (Table 1), indicating that under these conditions measuring DNA synthesis was an accurate measure of proliferation. As Fig. 1 shows, however, initiation of DNA synthesis in density-inhibited 3T3

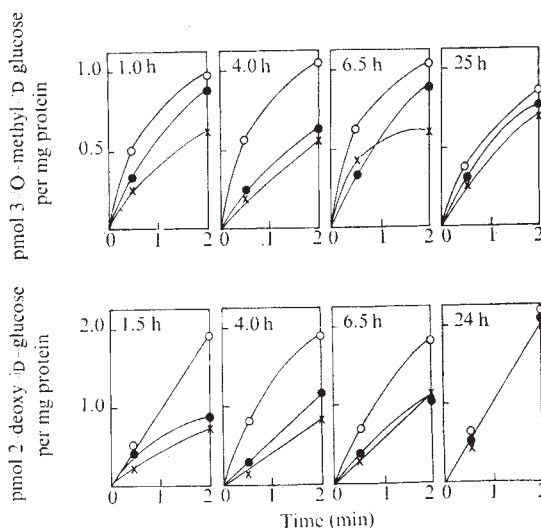


Fig. 1 Time course of 3-O-methyl-D-glucose and 2-deoxy-D-glucose uptake by control density-inhibited 3T3 cultures (●) and parallel cultures treated with 5% serum (○), or 3 μM cortisol (×). Times in hours refer to times after serum or cortisol treatment. Hexose uptake was measured by removing the growth medium, rinsing the cells once with PBS at 37°C and adding ³H-3-O-methyl-D-glucose (2 μCi ; 3.7 Ci mmol^{-1}) or ³H-2-deoxy-D-glucose (2 μCi ; 7.2 Ci mmol^{-1}) in 2.0 ml PBS. Cultures were incubated at 37°C for the times indicated on the abscissa. They were then immediately rinsed four times with PBS at 4°C. Less than 5% of the intracellular radioactivity was removed by this rinsing procedure. Further rinsing did not remove significant amounts of radioactivity from the cells. Acid-soluble radioactivity was extracted from the cells for one hour with 10% trichloroacetic acid (TCA) at 4°C, and measured in a liquid scintillation counter. Uptake measurements were corrected for radioactivity nonspecifically associated with the cells which was consistently less than 10% of the uptake value. Nonspecific association was determined by adding labelled hexose in PBS to cells at 4°C. This was immediately aspirated, and cultures were processed as described above for uptake measurements.

Table 2 Relative stimulation of DNA synthesis and hexose uptake by different lots of commercial serum

Addition	Thymidine incorporation		2-deoxy-D-glucose uptake	
	c.p.m. $\times 10^{-4}$ per mg protein	% control	pmol per mg protein	% control
None	0.11	100	0.90	100
C733308	1.05	950	1.42	159
A9219E	2.66	2420	1.14	128
R2034R	2.12	1930	1.07	119
R832101	2.51	2280	1.02	114
R636017	2.14	1950	1.01	113
W20720	2.47	2240	0.98	110

After growth to confluency, 5% fresh calf serum was added to cultures. Uptake of (^3H) 2-deoxy-D-glucose ($0.5 \mu\text{Ci ml}^{-1}$; 7.2 Ci mmol^{-1}) was measured during a 1-min pulse 4 h after the addition of serum as described in the legend to Fig. 1. DNA synthesis was measured at 24 h¹⁰. Protein was measured as previously described¹⁹.

fibroblasts was not always accompanied by increased hexose uptake. Addition of fresh serum to a final concentration of 5% brought about an increase in uptake of both 3-O-methyl-D-glucose and 2-deoxy-D-glucose by 1 h. The increase was maximal by 4–6 h and declined to values characteristic of density-inhibited cells by 24 h. This result confirms earlier findings⁸. In contrast to serum, cortisol inhibits hexose uptake by certain cells^{13,14}. Indeed, Fig. 1 shows that $3 \times 10^{-6} \text{ M}$ cortisol, a concentration which initiated DNA synthesis and division similar to 5% serum (Table 1), actually brought about a small decrease in transport of both hexose analogues by density-inhibited 3T3 fibroblasts. Figure 2 shows that this transport decrease occurred with several cortisol concentrations which initiated DNA synthesis. Thus, an increase in hexose transport is not necessary for subsequent proliferation.

While 2-deoxy-D-glucose has been commonly used to measure glucose transport, it has the disadvantage of being phosphorylated⁵. Thus, the intracellular radioactivity represents the sum of the phosphorylated and unphosphorylated forms of this sugar, and altered rates of transport might actually reflect altered phosphorylating activity. Since we obtained similar results with 3-O-methyl-D-glucose, which is not phosphorylated⁵, the observed alterations appear to be a result of transport changes rather than phosphorylation.

To probe further the relationship between initiation of proliferation and increased hexose transport, we added various levels of fresh serum (0.025% to 25%) to density-inhibited 3T3 fibroblasts and measured 3-O-methyl-D-glucose uptake, DNA synthesis and cell number. The dose-response curves in Fig. 3 show that fresh serum added to 0.5% caused a maximal increase in hexose transport but no significant increase in DNA synthesis or cell number.

As Fig. 3 shows, higher concentrations of serum brought about increases in DNA synthesis and cell number which were roughly proportional to serum concentration. These higher concentrations caused no additional increase in hexose uptake. Thus, an early increase in hexose uptake is not sufficient by itself to initiate proliferation of density-inhibited 3T3 fibroblasts.

Additional experiments indicated that serum might contain multiple factors which affect growth and hexose uptake and that the relative activities of these factors might vary in different lots of serum. For example, dose-response studies, like those shown in Fig. 3, revealed for some lots of serum parallel increases in hexose uptake, DNA synthesis and cell number (data not shown). In addition, the relative stimulation of DNA synthesis and hexose uptake varied with different commercial serum lots (Table 2). In fact, the lot which produced the greatest stimulation of hexose uptake had the smallest effect on DNA synthesis. Moreover, various pure or partially purified fractions of serum had differential effects on hexose uptake and DNA synthesis (Table 3). Addition of fetuin (0.5 mg ml^{-1}) produced a significant stimulation of hexose uptake but had no effect on DNA synthesis. As previously noted, cortisol stimulated DNA synthesis but not hexose uptake. Addition of insulin produced a graded increase in both responses (Table 3).

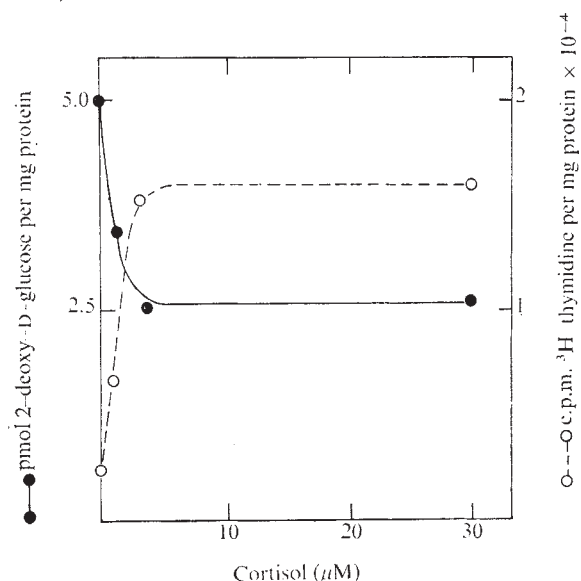


Fig. 2 Effect of cortisol concentration on hexose uptake and DNA synthesis by density-inhibited 3T3 cells. Uptake of 2-deoxy-D-glucose (●) was measured 4 h after cortisol addition as described in the legend to Fig. 1. DNA synthesis (○) was measured 24 h after cortisol addition as previously described¹⁰.

Table 3 Stimulation of DNA synthesis and hexose uptake by fetuin, cortisol, and insulin

Addition	Thymidine incorporation		3-O-methyl-D-glucose uptake	
	c.p.m. $\times 10^{-4}$ per mg protein	% control	pmol per mg protein	% control
I None	$1.89 \pm 0.15^*$	100	0.98 ± 0.19	100
0.5 mg ml ⁻¹ fetuin	$1.96 \pm 0.15^*$	103	1.61 ± 0.18	166
10% serum	$4.06 \pm 0.19^*$	214	2.56 ± 0.33	264
II				
$3 \times 10^{-6} \text{ M}$ cortisol	$1.10 \pm 0.22^*$	100	1.48 ± 0.06	100
	$2.38 \pm 0.07^*$	216	1.43 ± 0.11	97
III None	$0.25 \pm 0.03^\dagger$	100	1.50 ± 0.20	100
0.5 ng insulin per ml	$0.43 \pm 0.08^\dagger$	172	1.82 ± 0.04	121
200 ng insulin per ml	0.80 ± 0.13	320	2.16 ± 0.04	144

Fetuin, cortisol or insulin were added to confluent cultures and uptake of 3-O-methyl-D-glucose ($0.5 \mu\text{Ci ml}^{-1}$; 3.7 Ci mmol^{-1}) was measured during a 1-min pulse 4 h later as described in the legend to Fig. 1. DNA synthesis was measured as previously described¹⁰.

* Cultures labelled for 15 min 24 h after additions.

† Cultures labelled for 6 h from 21 to 27 h after additions.

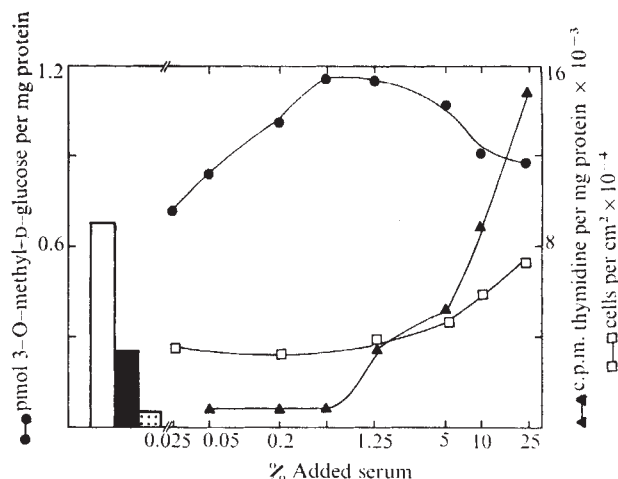


Fig. 3 Effect of concentration of added fresh serum on 3-O-methyl-D-glucose uptake (●), DNA synthesis (▲) and cell number (□). 3T3 cells were grown to confluency in medium containing 10% calf serum. Fresh serum was then added to the concentrations indicated on the abscissa. Hexose uptake was measured as described in the legend to Fig. 1 during 1 min incubation 4 h after addition of fresh serum. DNA synthesis was measured at 24 h (ref. 10) and cell number was monitored at 72 h by counting in a haemocytometer. Vertical bars show control values for density-inhibited cultures: plain bar, hexose uptake; solid bar, cell number; stippled bar, DNA synthesis.

These results demonstrate that increased hexose uptake is neither a necessary nor sufficient event for proliferation of density-inhibited 3T3 cells. Cortisol stimulates DNA synthesis and cell division in the absence of an early increase in hexose transport, and addition of fetuin or low concentrations of serum produce increased hexose uptake without subsequent proliferation. The conclusion that increased hexose uptake and proliferation may be unlinked is consistent with the findings of Gazdar *et al.* that treatment of mouse sarcoma virus-transformed BALB/c 3T3 cells with dibutyryl cyclic AMP and theophylline sharply inhibited cell growth yet stimulated glucose uptake¹⁵. In addition, Rubin and Fodge have suggested that 2-deoxy-D-glucose uptake merely reflects the activity of the glycolytic pathway which is activated after stimulation of proliferation¹⁶.

Early transport increases for phosphate and uridine have also been implicated in the initiation by fresh serum of proliferation of density-inhibited fibroblasts^{17,18}. In addition, Holley has shown that 3T3 cells can be arrested in G₁ (or G₀) by limiting phosphate in the medium, and that adding back phosphate leads to reinitiation of DNA synthesis³. De Asua *et al.* showed that addition of phosphate to quiescent 3T3 cells brought about a decrease in intracellular cyclic AMP levels and was required for full activation of uridine transport by serum¹⁸. They suggest that the increase in phosphate transport is a primary event in the reinitiation of growth. Cunningham and Pardee, however, showed that certain serum fractions, obtained by gel filtration on Sephadex G-200, initiated DNA synthesis in density-inhibited 3T3 fibroblasts but did not produce an increase in phosphate uptake. Moreover, fractions which increased phosphate uptake did not initiate DNA synthesis¹⁷. Thus, the early increase in phosphate transport is neither necessary nor sufficient for fibroblast proliferation brought about by fresh serum. We are probing further the possible involvement of changes in phosphate uptake in growth control processes.

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Dual mechanism for the action of cholesterol on membrane permeability

CHOLESTEROL, which is a widespread component of the plasma membrane, has a substantial influence on the permeability properties of lipid bilayer¹⁻³ and cell membranes⁴. Although the effects of cholesterol on membrane structure are increasingly well understood⁵ the mechanisms by which cholesterol modulates membrane permeability are obscure, mainly because it is difficult to deduce permeability properties from the knowledge of membrane structure alone.

We describe here quantitative⁶ determination of the influence of cholesterol on those variables of membrane structure—electric potential, chemical partition and rate of ionic transfer—which directly govern ionic permeability. The method used⁶ compares the ionic conductances induced by positively charged lipophilic species to those induced by negatively charged lipophilic species. Such probing of monolein membranes shows that although cholesterol somewhat decreases the rate of ionic transfer in these membranes, its predominant effect on ionic permeability arises by increasing the electric potential within the membrane interior, presumably by altering the strength and orientation of dipolar groups present at the membrane surface, since charged groups are absent.

The electrical conductance induced in Mueller and Rudin type⁷ bilayers by the lipophilic anions tetraphenylborate (TFB⁻) and carbonylcyanide *m*-chlorophenylhydrazine (CCCP⁻) as well as the lipophilic cations tetraphenyl phosphonium (TFP⁺) and 3,3'-dipropylodixadicarbocyanine iodide—diO-C₅-(3) (CC₅⁺)⁸ was characterised as a function of the

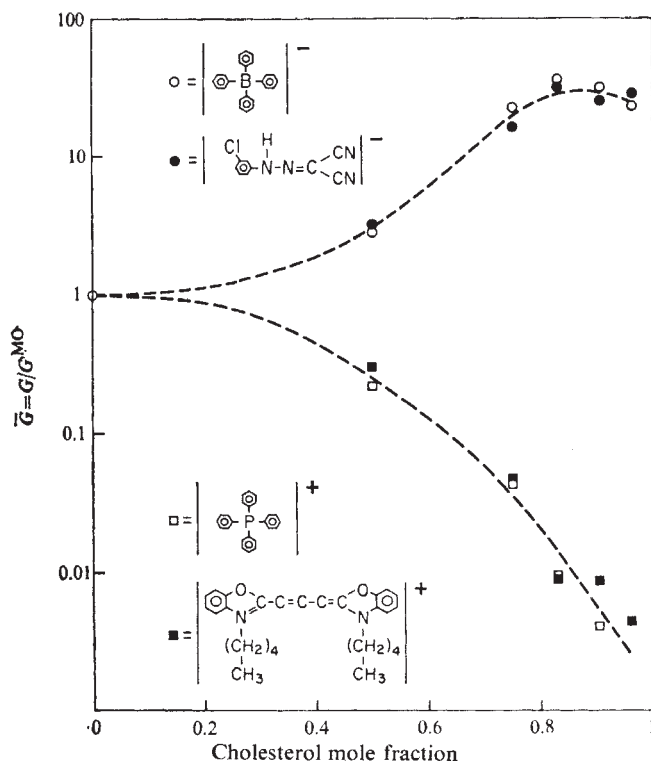


Fig. 1 Opposite effect of cholesterol on relative conductance of lipophilic cations and anions. Ordinate: relative conductance on a logarithmic scale. Abscissa: mole-fraction of cholesterol in the membrane-forming monoolein cholesterol solution. The total lipid concentration was 25 mM in decane. $\circ$, TFB $^-$; $\bullet$, CCCP $^-$; $\square$, TFP $^+$; $\blacksquare$, CC $_5^+$. For TFB $^-$ equilibrium values of the relative conductance were obtained by rapid conductance measurements⁹. The supporting electrolyte was 1 M NaCl. The solutions were buffered only for CCCP, at pH 6.0 with phosphate.

cholesterol-monoolein mole-fraction in the membrane-forming lipids (25 mM in decane) using previously described techniques¹. The measurements were made on membranes separating aqueous solutions of identical composition, in the limit of small applied d.c. potentials (± 10 mV) for which conductances are voltage-independent. Membrane conductances were independent of the type (NaCl or KCl) or ionic strength (10^{-2} M to 1 M) of the supporting electrolyte, confirming the expected absence of charged polar head groups in the monoolein-cholesterol bilayers⁶. For each of the lipophilic ions, the relative conductance, $\bar{G} = G/G^{MO}$, (defined as the ratio between the conductance of a cholesterol-rich membrane, G , and that of a pure monoolein membrane, G^{MO} , formed in a solution of the same composition) was found to be independent of the pH and of the lipophilic ion concentration over a wide range.

Figure 1 plots relative conductances for CCCP $^-$, TFB $^-$, TFP $^+$, and CC $_5^+$ as a function of increasing cholesterol-monoolein mole-fraction in the membrane-forming lipids. The most notable feature of Fig. 1 is the opposite effect of increasing cholesterol mole-fraction on the conductance of lipophilic anions and cations: the conductance for anions (CCCP $^-$ and TFB $^-$) is increased about 30-fold while the conductance for cations (TFP $^+$ and CC $_5^+$) is decreased about 100-fold. Moreover, an increase in the cholesterol mole-fraction has the same effect, within the experimental accuracy, on ions of the same charge in spite of the differences in the chemical structure and molecular shape and size of these ions (compare CCCP $^-$ with TFB $^-$ or TFP $^+$ with CC $_5^+$). In contrast, an increase in the cholesterol mole-fraction has an opposite effect on ions with opposite charges in spite of the identical structure of these ions (compare TFB $^-$ with TFP $^+$). Evidently it is the charge, and not the detailed chemical properties or molecular size, that determines the way in which conductance is altered by cholesterol. The lipophilic ions appear to 'sense' an increasingly positive

electrostatic potential in membranes of increasing cholesterol content. Furthermore, the opposite changes seen for cations and anions in Fig. 1 imply that the membrane conductance is influenced more by this potential than by changes in membrane fluidity, which should decrease the conductance for cations and anions.

To analyse quantitatively the data of Fig. 1, consider for a lipophilic ion of valance z , the theoretical expression of the relative conductance, $\bar{G}$, written as a function of $\Delta\psi_0 = \psi_0 - \psi_0^{MO}$, $\bar{k} = k/k^{MO}$ and $\Delta\mu^0 = \mu^0 - \mu_0^{MO}$ which respectively denote the change in the electrostatic potential (ψ_0), in the rate of ionic transfer (k) and in the chemical free energy of partition (μ^0) relative to a monoolein membrane (superscript MO)^{6,9}.

$$\bar{G} = G/G^{MO} = [\exp(-zF\Delta\psi_0/RT)] [\bar{k} \exp(\Delta\mu^0/RT)]$$

The two factors on the right-hand side of this equation have a simple physical meaning. The first factor describes the effect of the surface potential on membrane conductance. The second factor, which is the intrinsic conductance change that would be measured if there were no surface potential change, describes the effect of diffusion and chemical partition on membrane conductance. These two factors can be evaluated separately from the relative conductance for cationic ($\bar{G}^+$) and anionic ($\bar{G}^-$) species if the second factor depends on lipid composition in the same way for cationic and anionic species.

$$\exp(F\Delta\psi_0/RT) = (\bar{G}^+/\bar{G}^-)^{-1/2}; \bar{k} \exp(\Delta\mu^0/RT) = (\bar{G}^+/\bar{G}^-)^{1/2}$$

The data of Fig. 1 show that, within experimental accuracy, a single relative conductance, $\bar{G}^+$ and $\bar{G}^-$ respectively, describes the effect of cholesterol on different cationic and anionic species. This, together with the fact that TFB $^-$ and TFP $^+$ have the same molecular structure, implies that the condition for separating surface potential and intrinsic conductance is satisfied for the four ions of Fig. 1.

Figure 2 plots calculated values of relative surface potential (upper part) and intrinsic conductance change (lower part) as a function of cholesterol-monoolein mole fraction. The upper part of Fig. 2 shows that in accordance with intuitive expectation the electrostatic potential increases in the membrane interior as the cholesterol-monoolein mole-fraction is increased. This surface potential increase is large; it has a maximal value of 110 mV for predominantly cholesterol bilayers compared with pure monoolein bilayers and it accounts for most of the observed permeability changes. Since the surface-potential is independent of salt concentration, and furthermore, since the lipid has no ionisable polar head groups (that is, there are only —OH

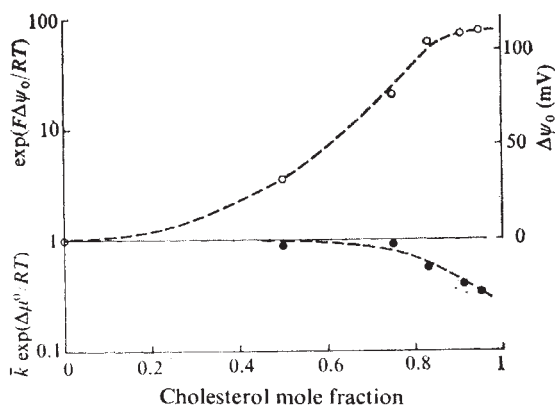


Fig. 2 Relative contributions of dipolar surface potential and intrinsic conductance changes to the effect of cholesterol on the membrane conductance induced by the lipophilic ions CCCP $^-$, TFB $^-$, TFP $^+$ CC $_5^+$ in monoolein membranes. The experimental data of Fig. 1 were analysed according to the procedure outlined in the text. Averaged values of the relative conductance for CCCP $^-$ and TFB $^-$ were used as $\bar{G}^-$; averaged values of the relative conductance for TFP $^+$ and CC $_5^+$ were used as $\bar{G}^+$.

groups, which are neutral at the pH of the experiments), the change in the surface potential must have its origin in changes in the orientation, strength and packing density of molecular dipoles at the membrane surface.

The existence of such dipolar surface-potential changes is not unreasonable^{10,11}. Indeed, about 90 mV difference of the correct polarity is found between compensation potentials of cholesterol (near 390 mV)¹² and monoolein (near 300 mV)¹⁰ monolayers spread at the air-water interface. Since a monolayer can be viewed as one half of a bilayer, the monolayer data lend independent support to the surface potentials deduced here solely on the basis of bilayer measurements, although care must be taken in making such comparisons since compensation potentials are steeply dependent on the extent to which the monolayer is compressed.

The lower part of Fig. 2 shows that cholesterol has a significant but small effect on intrinsic conductance compared with its main effect, caused by changes in surface potential (compare the left-hand scales on the upper and lower part of Fig. 2). Current-voltage measurements (that is, an $\exp(FV/2RT)$ type dependence of the current on voltage) indicate that the peak of the energy barrier¹³, and therefore the rate-limiting step for the permeation of CCCP⁻, TFB⁻, TFP⁺, and CC₅⁺, is located in the middle of the membrane. The small effect of cholesterol on intrinsic conductance shows, therefore, that the fluidity of the middle of the membrane is not much altered by cholesterol. This supports Rothman and Engelman's⁵ conclusion that cholesterol decreases fluidity only near the membrane surface and not in the membrane interior.

It should be emphasised that the influence of cholesterol on intrinsic conductance is not always small. Preliminary experiments indicate that cholesterol decreases substantially (that is, about 100-fold) intrinsic conductance for sodium complexes of the neutral carriers, valinomycin, nonactin and triactin. Current-voltage measurements (that is, an $\exp(FV/2.8RT)$ type dependence of the current on voltage) indicate that the energy barrier is trapezoidal¹³ and therefore the rate-limiting step for the permeation of these complexes is located nearer to the membrane surface, where cholesterol is expected to have its maximal effect on fluidity.

Probing of cholesterol-containing monoolein bilayers reveals that cholesterol modulates membrane permeability by two distinct mechanisms: by altering (1) the electrical potential difference across the membrane-solution interface, thereby changing the partition of charged species into the membrane, and (2) the fluidity of the membrane interior, thereby changing the rate of ionic transfer and the chemical partition of the solute in the membrane. The first mechanism is expected to act in the same way on the permeability of changed species of widely different molecular structure. In contrast, the second mechanism is expected to act differently on the permeability of different ionic species depending on the structural details of the membrane and of the ion. For CCCP⁻, TFB⁻, TFP⁺ and CC₅⁺ the second mechanism has only a small influence. For typical ion carriers, such as valinomycin, nonactin and triactin, its influence appears to be more prominent.

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Collagenase activity in experimental hepatic fibrosis

THE collagen fibres newly formed in experimental hepatic fibrosis and pre-cirrhosis are resorbed when the noxious stimuli have been removed¹. In investigating the mechanism of genesis and resorption of collagen fibres in experimental hepatic fibrosis^{2,3} we have found using electron microscopy that the collagen fibres are degraded extracellularly and partly engulfed by Kupffer cells and/or macrophages^{4,5}. Within these cells digestion of collagen fibrils or degraded collagen fibrils may occur in lysosomes⁶.

Native collagen is resistant to all known proteolytic enzymes except collagenase, which is operative at neutral pH under physiological conditions. The first animal collagenase was isolated^{7,8} from the culture fluid of tadpole skin. Subsequently, collagenase was found in the culture fluid of many kinds of tissues, such as skin, postpartum rat uterus, bone, human gingiva, cornea and synovium, and in the synovial fluid from patients with rheumatoid arthritis. Collagenase has not yet been definitively observed in the liver of either animals or humans¹⁰. Here we report the presence of collagenase in rat liver and increased collagenase activity in experimental hepatic fibrosis induced by carbon tetrachloride.

Fifty female Wistar strain rats, with an average weight of 150 g, received subcutaneous injection of 0.3 ml of 50% CCl₄ in olive oil per 100 g body weight, twice a week. Ten control rats were not treated. Nine to twelve rats were killed at 3, 6 and 20 weeks after the initial administration of CCl₄. Diced specimens (2 × 2 mm) of rat liver were removed under sterile conditions and transferred immediately to 105 mm Petri dishes with sterile mammalian Tyrode's solution to which had been added 150 U penicillin ml⁻¹ and 140 µg streptomycin ml⁻¹. They were washed repeatedly in mammalian Tyrode's solution for 24 h until they could be implanted on collagen substrate.

Salt-extracted collagen was prepared according to the method of Kang *et al.*¹¹. The skin from young, growing Wistar strain rats was used as the source of collagen. The purity of salt-extracted collagen was confirmed by disc electrophoresis and analysis of amino acid composition (manuscript in preparation). The assay method was similar in principle to that devised by Gross and Lapiere⁷. Two hundred and fifty microlitres of 0.1% collagen in cold mammalian Tyrode's medium was poured into microdishes 12 mm in diameter and 3 mm deep, and incubated at 37°C for at least 3 h. The reconstituted collagen gels were formed and were not digested by 50 µg of either trypsin, papain or pepsin (manuscript in preparation). Diced specimens of rat liver were placed on the rigid opalescent gel and incubated at 37°C for 72 h with mammalian Tyrode's solution in the outer ring (21 mm internal diameter and 5 mm deep) in a moist chamber containing 90% O₂ and 10% CO₂. The lysis area around the explant was observed daily and the sterility of the culture was frequently confirmed.

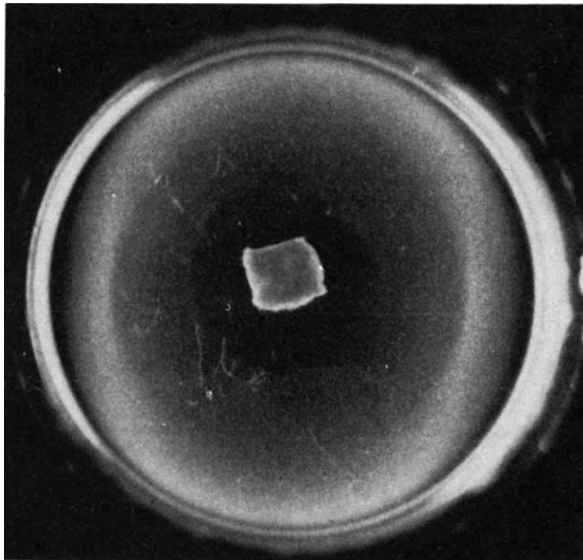


Fig. 1 Culture of rat liver treated with CCl_4 for 6 weeks showing area of lysis around explant (magnification $\times 5.8$).

In the ten non-treated rats, two livers demonstrated weak activity, five livers minimal activity and another three livers did not show appreciable lysis. Thus untreated rat livers have weak collagenolytic activity.

Microscopically the rat livers treated with CCl_4 for 3 weeks showed slight to moderate periportal fibrosis. Six weeks after the initial administration of CCl_4 , fibrous septa were formed connecting portal tracts with the central canal containing a tributary of the hepatic vein. After 20 weeks, the rat liver showed the typical features of cirrhosis including regenerative nodules. Collagenolytic activity increased slightly in the initial stage of hepatic fibrosis and markedly in the extensive fibrosis. Figure 1 illustrates lysis of the collagen substrate surrounding an explant of rat liver treated with CCl_4 administration for 6 weeks. The collagenolytic activity in the irreversible cirrhotic stage was poor compared with the activity in the rat liver treated with CCl_4 for 6 weeks.

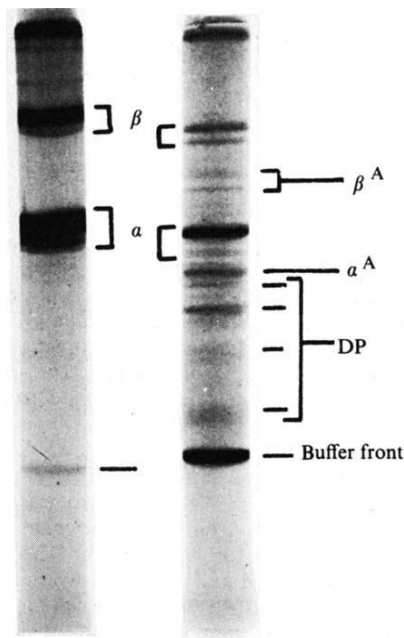


Fig. 2 Acrylamide gel electrophoresis patterns of products from incubations of collagen with rat liver treated with CCl_4 for 6 weeks (right) and without explant (left); α , monomer; β , dimers; α^A , β^A , degradation products of α and β respectively; DP, degradation products of lower molecular weight.

Acrylamide gel electrophoresis patterns of products from incubations of the collagen gel with rat liver treated with CCl_4 for 6 weeks are shown in Fig. 2. The control pattern consists of monomers (α), dimers (β) and higher molecular weight species. Incubation of collagen with the pre-cirrhotic rat liver at 35°C resulted in discrete products of α and β : α^A and β^A and degradative products of lower molecular weight which are similar to those seen with other animal collagenases^{12,13}. Repeatedly frozen and thawed rat liver tissue failed to induce visible lysis while living tissue from the same rat caused appreciable lysis; collagenolytic activity was inhibited by addition of sodium EDTA to a final concentration of 0.01 M (manuscript in preparation). Neither aerobic nor anaerobic bacterial contamination was detected in gels where lysis was seen.

Thus the increased collagenolytic activity observed in experimental hepatic fibrosis must be due to collagenase. The proliferation of collagen fibres must be because collagen is synthesised more rapidly than it is resorbed. The discontinuation of toxic stimuli presumably results in increased collagenolytic activity to resorb the excessive collagen fibres. The mechanism of hepatic fibrogenesis in human beings may be more complicated; we shall investigate it from the viewpoint of collagen metabolism in the liver.

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Glutamate metabolism in the frog retina

THE existence of a compartmented metabolism of glutamate in cerebral cortex is well established^{1,2}. When, for example, glutamate, acetate or γ -aminobutyric acid (GA) are metabolised by the tissue the specific activity of the glutamine formed exceeds that of the total tissue glutamate pool. As the immediate precursor of glutamine is glutamate, the implication must be that there is more than one pool of the latter. A model consisting of a minimum of three compartments seems most compatible with available data and compartment 1, from which glutamine is rapidly formed, is thought to be located in glial cells^{1,2}.

Why metabolism within the central nervous system is organised in this way is not certain but it has been proposed² that it may relate to the role of GABA as an inhibitory neurotransmitter—the suggestion being that GABA, when released

by neurones, is taken up by the glia, replacement carbon atoms then being returned to the neurones in the form of glutamine.

There is evidence that GABA may function as a neurotransmitter in the vertebrate retina^{3,4}. Wide species differences exist however⁴. In particular, autoradiography has shown that, in mammalian retinæ, exogenously applied GABA is accumulated into glial cells⁵⁻⁷; in the rabbit retina amacrine cells are also labelled⁷⁻⁹. In contrast, in lower order vertebrates such as goldfish, frog, pigeon and chicken, GABA goes solely into neurones¹⁰⁻¹². There are insufficient data from which to evaluate fully the role of GABA in mammalian and other retinæ but the above results imply fundamental differences in the way that the two groups handle the amino acid.

More than one pool of glutamate is present in rat retina¹³ and, as in brain, glutamate, aspartate, acetate, GABA, leucine and bicarbonate are all effective precursors of glutamine by way of a 'small' glutamate compartment. Because of the species differences in GABA uptake into retinal tissue and the postulated connection between glutamate compartmentation and the functional organisation of GABA, we thought it of interest to know how glutamate is metabolised in the frog retina. We have therefore investigated the metabolism of labelled acetate, glutamate and glucose in this tissue and have identified the cells accumulating ³H-glutamic acid by light microscope autoradiography⁶.

All frogs (*Rana temporaria*) were dark adapted overnight to free the neural retina from extensive contact with the pigment epithelium. The retinæ were then dissected out¹² under normal room lighting and incubated in 20 ml specimen bottles. At the end of the incubation period the labelled amino acids were extracted from the tissue and processed by a modification of the method described by Starr¹³. The concentrations of the amino acids present in the retina, after control incubations in the presence of unlabelled substrate, were determined using double label dansylation^{14,15}.

The results are detailed in Table 1. With glutamate or acetate as substrate, the specific activity (RSA) of glutamine relative to glutamate, after 30 min *in vitro* incubation, was greater than unity. In contrast, when glucose was the substrate, a normal precursor/product relationship was observed and the RSA was less than one. Thus a compartmentation of glutamate metabolism, qualitatively similar to that in the rat retina¹³, is present in the frog retina also. In addition the results imply that, as in other areas of the CNS, glutamate produced from glucose passes into the glutamate pool(s) from which glutamine is formed.

Table 1 Formation of aspartate, glutamate, glutamine and GABA from labelled precursors

Amino acid	U- ¹⁴ C-glucose		I- ¹⁴ C-acetate		U- ¹⁴ C-glutamate		nmol per retina ± s.e.m.
	SA	RSA	SA	RSA	SA	RSA	
Aspartate	58.3	0.28	67.6	0.29	369	0.13	16.6 ± 2.1
Glutamate	205	1.0	231	1.0	2757	1.0	40.4 ± 0.7
Glutamine	135	0.66	372	1.61	8952	3.25	23.7 ± 1.0
GABA	159	0.77	94	0.40	495	0.18	28.6 ± 0.6

Retinæ were preincubated for 15 min at 25° C in 2 ml oxygenated bicarbonate Ringer¹² containing 50 mg% glucose and then incubated for 30 min in the presence of D-U-¹⁴C-glucose (2.5 µCi ml⁻¹; 268 mCi mmol⁻¹), I-¹⁴C-sodium acetate (6.7 µCi ml⁻¹; 56 mCi mmol⁻¹) or L-U-¹⁴C-glutamic acid (2 µCi ml⁻¹; 260 mCi mmol⁻¹). After washing, free amino acids were isolated from the retinæ and the extracts were evaporated to dryness, taken up in 100 µl water and 30 µl taken for separation on Whatman No. 1 chromatography sheets (descending)¹³. The solvent was saturated aqueous phenol. The amino acids were separately eluted with 1.0 ml water and the radioactivity estimated by liquid scintillation counting¹². Standard quantities of glutamate, aspartate, glutamine and GABA, added to retinal homogenates, were processed and recoveries of 67–95% obtained. Experimental values were corrected accordingly. Endogenous amino acids were estimated by double label dansylation as previously described¹⁵.

SA denotes d.p.m. per µmol amino acid × 10⁻³ and RSA the specific activity relative to glutamate.

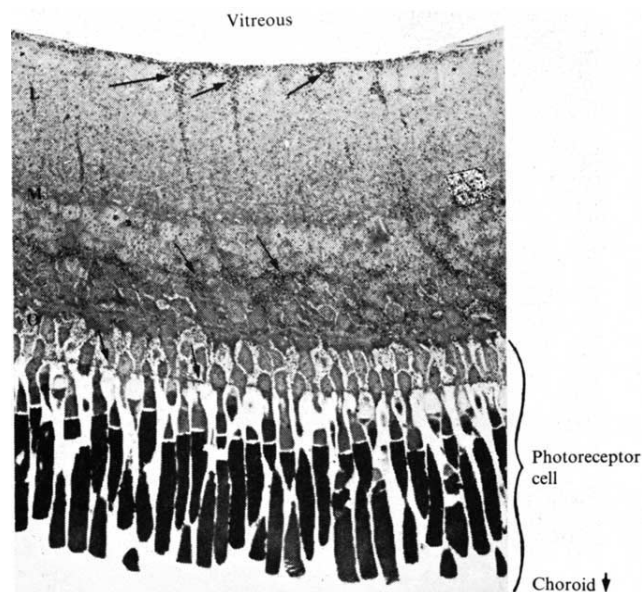


Fig. 1 Light microscope autoradiograph of frog retina showing the incorporation of ³H-glutamic acid into all regions of the Müller (glial) cells. Activity can be seen over the terminal expansions of these fibres at the inner limiting membrane (L), over their cell bodies (M) and over the outer limiting membrane (O)—the proximal termination of the Müller cell (×577.50). Retinæ were incubated for 30 min in the presence of 100 µCi ml⁻¹ DL-2-³H-glutamic acid (1.4 Ci mmol⁻¹) at 25° C. They were then fixed in glutaraldehyde followed by osmium tetroxide⁶ and processed for autoradiography. Ilford L4 photographic emulsion was used for the autoradiography and the slides exposed for 3 d.

A representative autoradiograph obtained from a frog retina preloaded with ³H glutamic acid (Fig. 1) shows there is a preferential accumulation by the Müller (glial) cells. Glutamate is also taken up by glia in both the rabbit⁹ and rat retina (Neal and White, personal communication). In our present study, with glutamate as substrate, labelled glutamine constituted 50% of the tissue radioactivity and it would seem, therefore, that these cells are the site of a glutamate pool that produces glutamine, certainly when glutamate is used as the labelled precursor. In current terminology this would correspond to the small glutamate compartment which in brain, as well, is thought to be located in the glial cells^{1,2}.

The formation of glutamine from glutamate within the Müller cells is compatible with the GABA–glutamine cycle proposed by Van den Berg². This cycle might occur in the rat retina where exogenous ³H-GABA is taken up by glial cells⁵. Such a scheme is, however, difficult to reconcile with the neuronal incorporation of ³H-GABA in the frog retina¹².

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Activity of CNS depressants related to hydrophobicity

SEVERAL theories of general anaesthesia account for the depression of neuronal excitability as a depression of membrane mechanisms underlying axonal conduction¹⁻⁴ or synaptic transmission⁵⁻¹⁶. The latter is more likely since general anaesthetics depress transmission in concentrations which do not block axonal conduction^{6,7,11,15,18}. In vertebrates, excitatory postsynaptic potentials (epsp) are invariably depressed^{12,15-20}, while under the same conditions inhibitory postsynaptic potentials (ipsp) and excitatory presynaptic potentials (resulting in presynaptic inhibition) are preserved (and prolonged)²⁴⁻²⁷. Using invertebrate preparations we found²⁸ that (1) pentobarbital selectively and reversibly depresses Na⁺-dependent epsps without affecting either Cl⁻ or K⁺-dependent ipsp, and (2) pentobarbital and other central nervous system (CNS) depressants selectively and reversibly depress postsynaptic excitation (induced by application of putative transmitters) which is coupled primarily to an increase in Na⁺ conductance without affecting postsynaptic inhibition (induced by application of putative transmitters) which is coupled to either Cl⁻ or K⁺ conductance increases. Selective depression of postsynaptic excitation would presumably lead to a decrease in the ratio of epsps to ipsp and thus account for much of the generalised decrease in neuronal activity during general anaesthesia. The observations by Meyer²⁹ and Overton³⁰ that general anaesthetic potency is directly proportional to the oil/water partition coefficient have prompted me to investigate the correlation between depressant activity and hydrophobicity. I report here that (1) depressant activity on invertebrate postsynaptic membranes is quantitatively correlated with hydrophobicity ($r=0.982$), and (2) analysis of published data from a vertebrate preparation indicates that the ratio of drug concentrations depressing axonal conduction and synaptic transmission equally is also quantitatively correlated with hydrophobicity ($r=0.96$).

Dose-response curves of the inhibitory effect of six CNS depressants (chloroform, chloralose, diphenylhydantoin-DPH, ethanol, pentobarbital and urethane) on the postsynaptic voltage response and conductance change of an identified neurone in *Otala lactea* in response to addition of 10⁻⁵ M acetylcholine (ACh) were constructed. The ACh response has an inversion potential of 0 to +10 mV and is dependent primarily on Na⁺ and secondarily on K⁺ (my unpublished observations). The dose-response curves approximately paralleled one another, suggesting that the agents each affected the ACh response through similar mechanisms. At least three and as many as ten curves were used to establish the concentration of an agent which inhibited the ACh response by 50%. The concentration producing 50% inhibition can be correlated quantitatively with the agent's hydrophobicity, as reflected by its octanol/water partition coefficient (Table 1, Fig. 1). Least

Table 1 Hydrophobicity and concentration of CNS depressant inhibiting ACh response on a molluscan neurone

Compound	log P*	Observed	log 1/C Calculated	Dev.
Ethanol	-0.32	0.70	0.85	+0.15
Urethane	-0.15†	1.23	1.04	-0.19
Chloralose	1.60	3.00	3.09	+0.09
Chloroform	2.00	3.16	3.56	+0.50
Pentobarbital	2.00	4.00	3.56	-0.44
Diphenylhydantoin	2.50	4.16	4.15	0.01

P, octanol/water partition coefficient; C, molar concentration.

* Obtained from ref. 47.

† Experimentally determined by C. Silipo and C. Hansch.

squares analysis of my data yielded equation (1) which relates the molar concentration, C, causing 50% depression of the ACh response to octanol/water partition coefficient, P

$$\log 1/C = 1.17 \log P + 1.22 \quad (1)$$

A statistical analysis, using F tests, gave:

$$\frac{n}{6} \frac{r}{0.982} \frac{s}{0.295} \frac{F_{ratio}}{F_{1,4}} = 125; F_{\alpha} = 0.0005 = 106$$

where n is the number of data points, r is the correlation coefficient, s is the standard error of the estimate, F_{ratio} for significance test with $F_{k, n-k-1}$ compared with that at a specific level of confidence, α , where k is the number of independent variables and n is the number of data points used. Thus depressant activity is positively and significantly correlated with hydrophobicity over a thousandfold range of hydrophobicity and activity for the differently structured compounds, implying that steric requirements for activity are minimal.

This depression of postsynaptic excitation in invertebrates complements similar observations in the vertebrate at neuromuscular junctions^{1,12,31-33} and on CNS neurones³⁴⁻³⁸. An increase in evoked transmitter release at neuromuscular junctions during exposure to barbiturates^{19,20} when epsp amplitude is reduced indicates that epsp depression here is due to a postsynaptic action. Generalised depression of postsynaptic excitation coupled primarily to Na⁺ (and secondarily to K⁺) conductance may thus account for much of the depression of neuronal excitability. Since, however, blockage of axonal conduction has been described in both invertebrates and vertebrates^{1-4,39}, I have investigated its importance by analysing data published by Larrabee and Posternak⁶. They examined

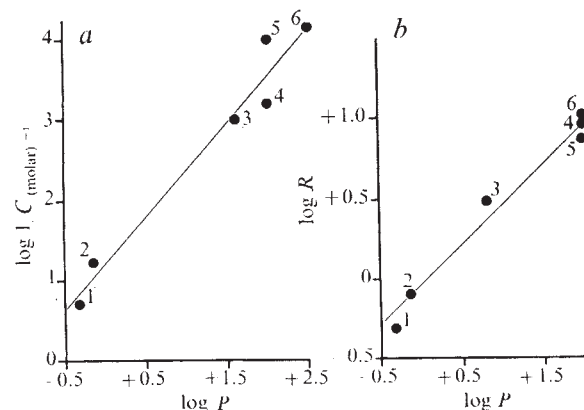


Fig 1 CNS depressant activity related to hydrophobicity. *a*, Log-log plot of inverse of molar concentration, C, of CNS depressants reducing ACh response on a snail neurone by 50% as a function of hydrophobicity, P, of compounds. 1, Ethanol; 2, urethane; 3, chloralose; 4, chloroform; 5, pentobarbital; 6, DPH. *b*, Log-log plot of ratio, R, of CNS depressant concentration depressing (by equal percentage) pre- and postsynaptically derived compound action potential in cat stellate ganglion as a function of hydrophobicity, P, of the agents. Data are from Larrabee and Posternak⁶. 1, Ethanol; 2, urethane; 3, ether; 4, chlorotone; 5, chloroform; 6, pentobarbital.

the effects of general anaesthetics on axonal conduction and synaptic transmission through the cat stellate ganglion. The concentration blocking the postsynaptic event by 50% correlated with hydrophobicity and least squares and statistical analysis of the data yield

$$\log 1/C = 1.21 \log P + 0.83 \quad (2)$$

and a correlation coefficient, $r = 0.96$. Equation (2) is close to that found in an analysis of data from the rabbit cervical ganglion⁴⁰ by Hansch and Dunn⁴¹ who obtained

$$\log 1/C = 1.11 \log P + 0.81 \quad (3)$$

and $r = 0.978$. Equations (2) and (3), calculated using data from vertebrate synapses, are similar to equation (1), the major difference lying in the intercepts, which reflect the sensitivities of both the systems to the drugs and of the drug effects to the assay. The similarity inslopes of the equations indicates a similar dependency of activity on hydrophobicity.

The concentration required to block the synaptically-derived compound action potential was compared with the concentration required to block the directly recorded (presynaptic) compound action potential by an equal percentage. The ratio of the concentrations, R , was then correlated with hydrophobicity, P (data in Table 2):

$$\log R = 0.49 \log P - 0.03 \quad (4)$$

A statistical analysis gave

$$\frac{n}{6} \frac{r}{0.96} \frac{s}{0.295} \frac{F_{ratio}}{F_{1,4}} = 262; F_{\alpha} = 0.000.5 = 106$$

Equation (4) allows one to predict the ratio of depression of synaptic transmission to depression of axonal conduction from hydrophobicity. Pentobarbital, chloroform and chloretone are about ten times and ether about three times more selective for blocking synaptic transmission than for blocking axonal conduction, while ethanol and urethane appear to depress both aspects of neuronal excitation equally. Possible mechanisms include (1) a decrease in transmitter release; (2) a decreased chemosensitivity of postsynaptic receptor; (3) a blockade of the conductance mechanism coupled to the receptor, and (4) a nonspecific alteration in postsynaptic membrane properties so as to decrease the probability of excitation occurring. An increase in transmitter release at vertebrate neuromuscular junctions by these agents has been reported^{19,20,42} in contrast to a decrease in release at motoneurons⁴³. (The latter may be due to an increase in presynaptic inhibition through effects on the primary afferent fibre terminals.) Nonspecific alterations in postsynaptic membrane properties do occur^{13,14} but at 10–100 times the concentration necessary to selectively block the receptor-coupled conductance mechanism in invertebrates and to produce effective general anaesthesia⁴⁴. If the blockade of synaptic transmission in this ganglionic preparation is related to the postsynaptic effects observed in invertebrate systems²⁸ and in vertebrate neuromuscular junctions^{19,20} where alterations in end-plate currents by general anaesthetics have been described^{45,46}, then depression of synaptic physiology in vertebrate

ganglia more likely reflects a depression of the conductance mechanism coupled to postsynaptic receptors.

In summary, analysis of data derived from invertebrate and vertebrate preparations indicates that (1) agents which depress CNS excitability selectively depress epsps in invertebrates which are predominantly Na^+ dependent; (2) depressant activity at a molluscan postsynaptic membrane is correlated with hydrophobicity ($r = 0.982$); (3) depression of a postsynaptically derived compound action potential is similarly correlated with hydrophobicity ($r = 0.96, 0.978$); and (4) the ratio of drug concentrations required to depress postsynaptic excitation and presynaptic axonal conduction equally is also positively correlated with hydrophobicity ($r = 0.96$). The results suggest that much of the depression of CNS excitability observed with those agents commonly used as general anaesthetics is due primarily to a selective depression of postsynaptic excitation through a postsynaptic mechanism and secondarily to a depression of axonal conduction by the more hydrophilic agents (ethanol and urethane). In addition, the mechanisms underlying the effects of general anaesthetics on presynaptic physiology in the vertebrate and the relative importance of presynaptic inhibition in the depression of CNS excitability by these agents requires further investigation. Finally, the roles played by hydrophobicity in determining whether the attack of these agents is directed either to voltage-independent (postsynaptic receptor-coupled) and/or to voltage-dependent (axonal) monovalent conductance mechanisms (and to presynaptic physiology) remains to be elucidated.

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Table 2 Hydrophobicity and ratio of CNS depressant concentration inhibiting pre- and postsynaptic compound action potential

Compound	$\log P^*$	Observed†	$\log$ Ratio Calculated	Dev.
Ethanol	-0.32	-0.30	-0.19	-0.11
Urethane	-0.15	-0.10	-0.10	0
Ether	+0.80	+0.48	+0.36	+0.12
Chloretone	2.0	+0.95	+0.95	0
Chloroform	2.0	+0.85	+0.95	-0.10
Pentobarbital	2.0	+1.00	+0.95	+0.05

* Obtained from ref. 47.

† Obtained from ref. 6.

P , octanol/water partition coefficient; C , molar concentration.

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Temperature-dependent interconversion of histamine H_1 and H_2 receptors in guinea pig ileum

HISTAMINE receptors mediating changes in gastric secretion are resistant to blockade by most antihistamines¹, and this led Ash and Schild² to propose two classes of receptors, H_1 typified by the system in guinea pig ileum or bronchi, and H_2 responsible for effects on atria and gastric secretion. This concept is supported by the demonstration that, in contrast to most antihistamines, a series of substituted imidazole compounds are competitive antagonists of the H_2 receptor with negligible action on H_1 (refs 3, 4).

During studies of the effects of changes in temperature on the blocking action of some competitive H_1 antagonists, we found that below 18° C promethazine ceased to be competitive and appeared to act non-competitively. Since promethazine can act as a non-competitive antagonist of the H_2 receptor in atria³ this raised the possibility of some transformation of the H_1 to the H_2 receptor at lower temperatures. We report here studies which support this view.

Isolated strips of the longitudinal smooth muscle of guinea pig ileum⁶ were set up in organ baths containing

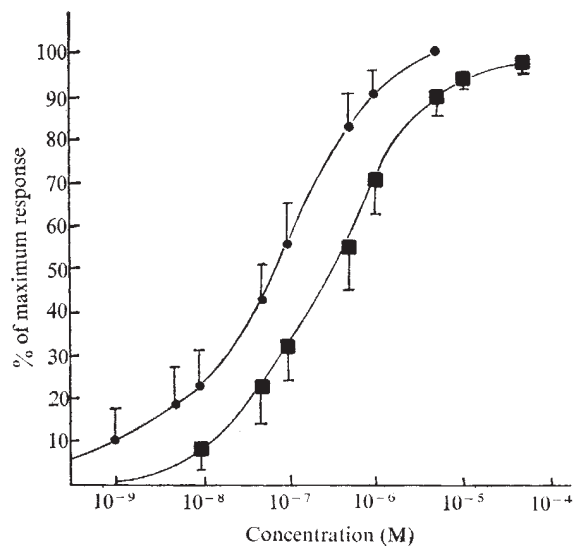


Fig. 1 Dose-response curves for histamine at 12° C. Bars represent standard error from five experiments. ●, Control; ■, after treatment with 7.5×10^{-5} M metiamide.

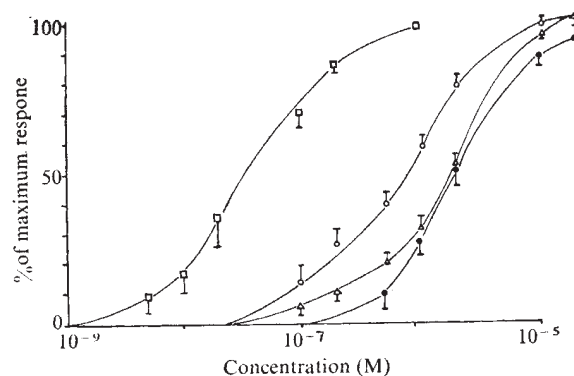


Fig. 2 Dose-response curves for histamine. Bars represent standard errors from six experiments. □, Control response to histamine at 37° C; △, after 5×10^{-8} M tripeleennamine at 37° C; ○, after 5×10^{-8} M tripeleennamine at 12° C; ●, after 5×10^{-8} M tripeleennamine and 7.5×10^{-5} M metiamide at 12° C.

Tyrod solution gassed with 95% O_2 :5% CO_2 and allowed to equilibrate for 1 h at 37° C. Contractions were recorded using either a frontal writing lever and a kymograph, or a linear motion transducer (Hewlett Packard 7DCDT) and a polygraph (Grass 5P1). Responses to histamine in this preparation were stable for several hours both at 37° C and 12° C, although some changes in shape of the dose-response curve were observed at lower temperatures. At 37° C the H_2 antagonist metiamide, at concentrations up to 2×10^{-4} M, had no effect on the responses to histamine, in agreement with the findings of Black *et al.*⁴. At 12° C 7.5×10^{-5} M metiamide produced a parallel shift in dose-response curve to the right (Fig. 1). Rewarming the tissue to 37° C resulted in some persistence of blockade, but after 1 h responses were similar to those obtained initially in the absence of metiamide.

To demonstrate that the effects were specific for the histamine receptor these experiments were repeated using acetylcholine as the agonist. Metiamide produced no antagonism of the responses to this agent at either 37° C or 12° C at concentrations up to 10^{-4} M.

The H_1 receptor antagonists usually have some action on H_2 receptors, but tripeleennamine is reported to have very high selectivity for H_1 (ref. 7). Tissues treated with 5×10^{-8} M tripeleennamine at 37° C showed the expected shift in dose-response curve to the right. At 12° C, and in the continued presence of tripeleennamine, some reversal of blockade occurred and this could be blocked again with metiamide (Fig. 2). As this recovery was unaffected by 10^{-6} M atropine it is unlikely to have arisen from any action of histamine on release of acetylcholine. No sensitisation to acetylcholine was observed at lower temperatures, after treatment with either atropine or tripeleennamine.

The ability of metiamide to antagonise the histamine-induced responses of this preparation at lower temperatures and the reduced ability of tripeleennamine to block contractions under these circumstances suggests that at 12° C some receptors were transformed from H_1 to H_2 . This is in many ways similar to the findings of Kunos *et al.*⁸ concerning the interconversion of the α and β receptors in heart, but we are unable, to present, as they did, evidence based on selective actions of agonists since selective H_1 and H_2 agonists were not available to us. Betazole is reported to be about ten times as active on sites regarded as H_2 receptors⁹, and it would thus be expected that the tissue would show increased sensitivity to betazole at lower temperatures. A small increase in sensitivity to betazole was observed in three of six experiments and this was antagonised with metiamide, but control experiments indicated that betazole shows marked tachyphylaxis particularly at

low temperatures, and this observation makes study of the H_1 to H_2 interconversion using this agonist difficult to evaluate.

In conclusion, our data suggest that at low temperatures some H_1 receptors of guinea pig ileum convert to a form similar to the H_2 receptor. The concept of a change in the properties of receptors as a result of changes in temperature is not unexpected in view of the concept of membrane phase transitions supported by enzyme studies¹⁰. Furthermore, Rocha e Silva¹¹ has suggested that the properties of the histamine receptor at temperatures above 20°C are different from those at lower temperatures.

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Astrocyte-specific protein and radial glia in the cerebral cortex of newborn rat

HERE we show that radial glia directing neuronal migration in the cerebral cortex during development can respond to injury with the production of a protein specific to astrocytes, and may thus be regarded as an early form of the mature astrocyte.

The migration of nerve cells from their site of origin in germinal zones to their final destination is a general rule in brain development, the granular layer of the hippocampus being the only possible exception. Migrating neuroblasts in the cerebellar and cerebral hemispheres are in close contact with radially oriented glial processes¹⁻³. Such close relationship suggests that glia play a role in directing radial migration of neurones through complex regions of the brain which contain bundles of nerve fibres in various orientations early in development. In the cerebellum, Bergmann glia provide the guidelines for neurones migrating inwards from the external granular layer¹. They persist in adult life as a distinct form of fibrous astrocytes with straight parallel processes crossing the molecular layer at right angles and reaching the surface of the cerebellum⁴. During development of the cerebral hemispheres the radially oriented glial cells directing the outward migration of neurones from the ventricular zone have been observed in many species by the Golgi method and more recently by electron microscopy in the monkey and the rat^{2,3}. These cells are not observed in mature brain and have been interpreted as spongioblasts (pluripotential glial precursors³), or as transient forms of astroglia trans-

forming into astrocytes of more typical appearance later in development^{2,5}.

The glial fibrillary acidic (GFA) protein is brain specific but not species specific and is selectively located in fibrous astrocytes^{6,7}. The appearance and distribution of neuroglia using immunofluorescence with GFA protein antibodies correspond to those described by Weigert⁴ with his method for fibrous astrocytes. By immunofluorescence, Bergmann glial processes transversing the external granular layer and molecular layer of the cerebellum are first demonstrated on the third postnatal day in the rat and mouse, thus before the beginning of neuronal migration in these species^{8,9}. In a similar study of astroglial differentiation in the rat cerebral hemispheres⁷, radial glial processes were only demonstrated in the granular layer of the hippocampus in the first weeks of postnatal development. Immunofluorescent fibres were first observed on the 18th embryonic day in the medial wall of the lateral ventricles and they had no radial orientation.

After injury the astrocytes become larger with increasing gliofibrillary content. In a study of the glial reaction during development, the brains of adult and newborn rats were stabbed unilaterally in the lateral frontal region. This was done with a scalpel through the intact skull in the parasagittal plane. Animals were killed at intervals ranging from 2 d up to 2 months. Air-dried coronal cryostat sections were used for immunofluorescence studies. Adjacent sections were stained with toluidine blue. GFA protein antisera previously obtained from New Zealand albino rabbits⁷ were used. The rabbits had been injected with human GFA protein purified from multiple sclerosis plaques, a severely gliosed tissue markedly enriched in GFA protein^{10,11}. The immunofluorescence test was carried out according to Coons indirect procedure, as previously described⁷. Control sections were incubated with preimmunisation sera and immune sera absorbed with GFA protein. GFA protein antisera and preimmunisation sera were used in 1/50 dilutions. At this titre essentially no fluorescein staining was seen in control sections. The fluorescein-labelled goat anti-rabbit globulin serum was used in 1/10 dilutions.

In toluidine blue stained sections the damage to the lateral frontal cortex was extremely limited and confined to the vicinity of the blade track. In the subjacent white matter the damage was more extensive and in newborn animals resulted in a necrotic and cavitating lesion bordering the frontal cortex on the medial surface of the hemisphere.

The astrocytic reaction to injury was studied by immunofluorescence with GFA protein antiserum in the frontal cortex. In the rat isocortex most astrocytes are protoplasmic and do not contain GFA protein in detectable amounts. In the adult animal immunofluorescence is essentially confined to the external glial membrane on the surface of the brain and to the glial membranes surrounding small arteries and veins (Fig. 1A). None of these structures is immunofluorescent in the newborn rat. A continuous external glial membrane is first observed by immunofluorescence on the 9th day, while the perivascular glial membranes form later (9-18 d) (ref. 7). In the adult rat 2 d after stabbing protoplasmic astrocytes had switched to GFA protein production and thus became brightly immunofluorescent with GFA protein antiserum (Fig. 1B). This change, still present 2 months after injury, was not confined to the vicinity of the wound but extended through wide areas of the cortex in the stabbed hemisphere. In newborn rats sacrificed 2 d after stabbing, at a time when no immunofluorescence is normally present, immunofluorescent fibres had appeared around the blade track in the lateral frontal cortex (Fig. 1C) and in the medial frontal cortex overlying the necrotic lesion in the white matter (Fig. 2). While in the lateral cortex these processes were irregularly oriented, in the medial cortex they had the appearance of thin parallel processes transversing the cortex at right angles to the surface. These radial processes had disappeared 13 d after injury. At that time immunofluorescent fibres had the same irregular orientation in the medial and lateral cortex. In 1-month-old

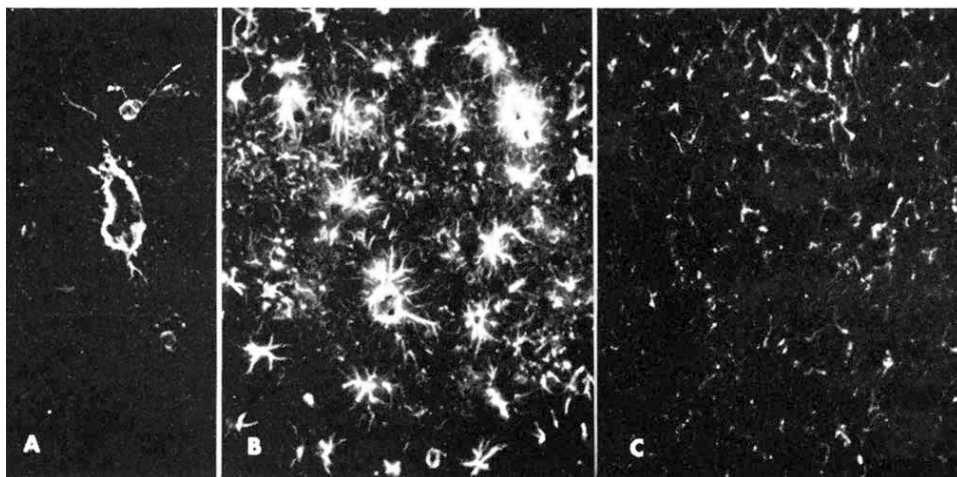


Fig. 1 Astrocytic response to injury in the middle layers of the rat frontal cortex 2 d after stabbing. In the normal adult rat (A) immunofluorescence with GFA protein antiserum is essentially confined to the glial membranes surrounding small arteries and veins. After stabbing (B) the protoplasmic astrocytes which normally do not stain become brightly immunofluorescent. This is an extensive reaction involving large areas of the stabbed hemisphere. In the newborn rat (C) the reaction is already present but less intense (no immunofluorescence is normally seen ($\times 14.5$) at this stage of development). Immunofluorescence with GFA protein antiserum.

animals which had been stabbed at birth, the reactive astrocytes in the cerebral cortex were identical to those observed in mature animals following the same type of lesion, that is they appeared as conventional astrocytes in Cajal preparations. The astrocytic reaction had become very extensive and was also present in the cortex of the unstabbed hemisphere.

Radial glial cells in the medial frontal cortex of the newborn rat behave like protoplasmic astrocytes in mature brain, that is, they are switched to GFA protein production as a response to injury. Radial glia may thus be considered as an early form of astrocytes that transforms into astrocytes of more conventional morphology in the first weeks of postnatal development. It may be noted that radial glia persist through adult life as the main type of supporting glia in lower vertebrates¹². As is the case with astrocytes in mammalian brain, radial glial cells in lower vertebrates may not contain GFA protein in detectable amounts. Thus, radial glia could be demonstrated by immunofluorescence in the optic tectum of the turtle, but not in the optic tectum of the goldfish and frog⁶.

It seems that astrocytic determination, as opposed to GFA protein expression, is an early phenomenon in development. According to Rakic (personal communication), some of the elongated columnar neuroepithelial cells spanning the entire thickness of the telencephalic wall, develop vascular attachments as early as 48 d after conception in the monkey (gestation time 165 d). The constraints imposed by the mesenchymal interaction are probably a major factor restricting the pluripotential neuroepithelium to the role of supporting glia (radial astrocytes). GFA protein expression occurs much later in the

course of normal development, most likely as a result of permissive tissue interactions.

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Fig. 2 Radial glia in the newborn rat (deep layers of the medial frontal cortex) 2 d after stabbing. Radial glia is not stained by immunofluorescence with GFA protein antiserum in normal development. W, injured white matter underlying the medial frontal cortex. Immunofluorescence with GFA protein antiserum ($\times 100$).

Apomorphine and morphine stimulate prostaglandin biosynthesis

AMONG the acute pharmacological effects of apomorphine and morphine are emesis, hyperthermia and hyperglycaemia^{1,2}. Because administration of prostaglandins also elicits each of these effects³⁻¹⁰, we have investigated whether apomorphine or morphine stimulates prostaglandin biosynthesis. Here we describe experiments showing that both drugs stimulate, although possibly in a somewhat different way, the prostaglandin (PG) synthetase of bull seminal vesicles.

Bull seminal vesicles, kept for not more than four weeks at -20°C , were homogenised for 1-2 min at 4°C in three volumes of 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. The strained homogenate was centrifuged at 600g for 15 min and the supernatant separated and stored under nitrogen at -20°C . Two different preparations of this supernatant were used in the course of these experiments, with comparable results. The incubation mixture consisted of 500 μl of the supernatant in 2 ml of 50 mM phosphate buffer, with or without apomorphine hydrochloride or morphine sulphate. The reaction was started by addition of sodium arachidonate (final concentration 61 μM) and the tubes were

aerated with gentle shaking. After 15 min, the reaction was stopped by adding 2 ml 0.2 M citric acid and the mixture was extracted with 16 ml ethylacetate. After centrifugation at 600g for 5 min, 11 ml of the ethylacetate layer was evaporated to dryness *in vacuo*, the residue dissolved in 500 μ l ethanol, and 100 μ l aliquots were diluted 50-fold in Krebs' solution. Volumes of not more than 0.25 ml of these aqueous ethanol extracts were assayed against reference PGE₂ on rat isolated stomach fundus strip, superfused at 5 ml min⁻¹ with Krebs' solution, containing mixed antagonists of acetylcholine, catecholamines, histamine and 5-hydroxytryptamine¹¹, and aerated with oxygen containing 5% carbon dioxide.

In some experiments, aliquots (125 μ l) of the ethanol solution were chromatographed in the A I system¹² on silica gel plates with PGE₂ and PGF_{2 α} markers. The areas corresponding to each prostaglandin were separately extracted with ethanol and the residues after evaporation were redissolved in 200 μ l ethanol and diluted 25-fold with water for bioassay. Assays were then performed against reference PGE₂ and PGF_{2 α} on rat isolated fundus strip and colon, superfused in series. The concentrations of drugs are expressed as those of active acid or base.

In control experiments, we found that addition of apomorphine (100 or 400 μ g ml⁻¹) or morphine (10 or 100 μ g ml⁻¹) to the boiled enzyme before incubation did not affect the blank assay values for total prostaglandin, indicating that the assay was not affected by drug being carried through the extraction process. In other control experiments, ³H-PGE₂ and ³H-PGF_{2 α} were incubated with unboiled or boiled enzyme for 15 min, extracted, separated by thin layer chromatography, and assayed. In this procedure, < 8% of the PGE₂ and no PGF_{2 α} were lost during incubation with unboiled enzyme preparation. We concluded that a very large part of the increases in net production of prostaglandins found in the presence of apomorphine or morphine could be attributed to increased prostaglandin biosynthesis and not to protection of prostaglandin from enzymatic breakdown.

PG synthetase was incubated with arachidonic acid in the absence of drug, or in the presence of different concentrations of apomorphine, and the yield of prostaglandin-like material assayed against PGE₂. After subtracting the amount of PG-like material found in control tubes containing boiled enzyme, the assay value was taken to represent PG produced by the action of synthetase during incubation. With unboiled enzyme in the absence of drug, a mean of 1,137 ng (s.e. $\pm$ 129) of prostaglandin was produced. When apomorphine (0.1–100 μ g ml⁻¹) was present, mean PG production exceeded the control value by 1.26-fold to 4.42-fold (Fig. 1). In the presence of apomorphine (1–100 μ g ml⁻¹), mean PG production sharply increased with increasing concentration of drug (slope, 1.64; s.e., $\pm$ 0.379, P < 0.001). A higher concentration of apomorphine (400 μ g ml⁻¹) inhibited PG production. From the mean concentration/response line it was calculated that 1.71 μ g ml⁻¹ (limits, 1.33–5.44) of apomorphine were required to increase the PG production by a factor of 1.5 (SC 50).

In similar experiments, morphine (0.01–100 μ g ml⁻¹) stimulated PG production by 1.21-fold to 2.17-fold (Fig. 2). Compared with apomorphine, morphine had a concentration/response line of low slope; but this was statistically significant (slope, 0.149; s.e., $\pm$ 0.045; P < 0.005). The SC₅₀ value for morphine was 1.59 μ g ml⁻¹ (limits, 0.0855–52.4).

In both experiments with each drug, in which prostaglandins produced were separated by thin layer chromatography before assay, apomorphine and morphine stimulated production of both PGE₂ and PGF_{2 α} . For example, in one experiment, apomorphine, at 100 μ g ml⁻¹, increased production of PGE₂ by 2.47-fold and that of PGF_{2 α} by 1.66-fold; and morphine, at 1 μ g ml⁻¹, increased production of PGE₂ by 1.30-fold and of PGF_{2 α} by 1.27-fold.

In three independent experiments, chlorpromazine hydrochloride and sodium acetylsalicylate were tested for their ability to inhibit the stimulation of PG synthetase induced by

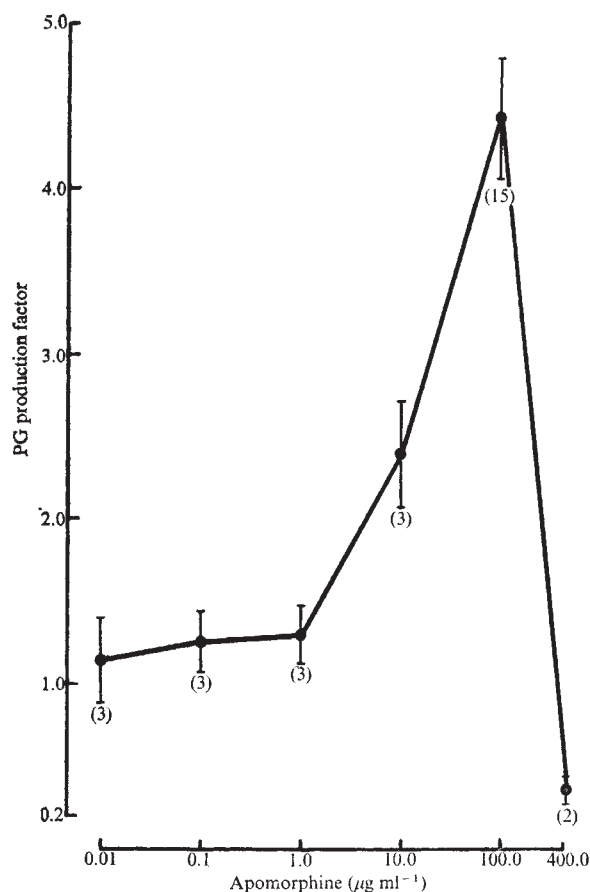


Fig. 1 Stimulation by apomorphine of the PG synthetase of bull seminal vesicles. The production factor is the factor by which prostaglandin production in the presence of apomorphine exceeded that in its absence in reference tubes in the same experiment. The results of 2–15 independent experiments are expressed as the mean production factor $\pm$ s.e. in brackets, the number of values used to calculate each mean.

100 μ g ml⁻¹ of apomorphine. Chlorpromazine, at 0.9, 9 and 90 μ g ml⁻¹, reduced apomorphine stimulation in a dose-related way, by mean percentages of 3.1, 25.8 and 60.5 respectively. Acetylsalicylate reduced PG production both in the presence and absence of apomorphine.

These experiments show that apomorphine and morphine stimulate the prostaglandin synthetase of bull seminal vesicles in the absence of the cofactors, glutathione and hydroquinone, that are usually added. Experiments now in progress show that such stimulation can also occur in the presence of these cofactors¹³. Other experiments in progress show that the stimulation by apomorphine and morphine of PG synthetase is not restricted to the enzyme from bull seminal vesicles, but occurs also with synthetase from rabbit brain¹⁴.

The low slope of the concentration/response relationship for morphine could arise from various causes; for example, the action of morphine could be limited by a factor independent of the drug concentration; or, again, morphine might activate processes with opposed concentration/response relationships. The difference in this respect from apomorphine may indicate a difference in the mechanism of action of morphine and apomorphine on this preparation.

Morphine is known to act centrally in producing emesis^{15,16}, hyperthermia¹⁷ and hyperglycaemia^{18,19}. Apomorphine, too, exerts these effects by acting on the brain (ref. 15 and W. Feldberg, unpublished). In all the experiments reported above, apomorphine and morphine stimulated prostaglandin biosynthesis at concentrations less than those attainable in the brain with tolerated doses of either drug^{20,21}. Prostaglandins are produced in the brain²², and E-type prostaglandins are known to act there to elicit fever^{7,8}. It is not known, however,

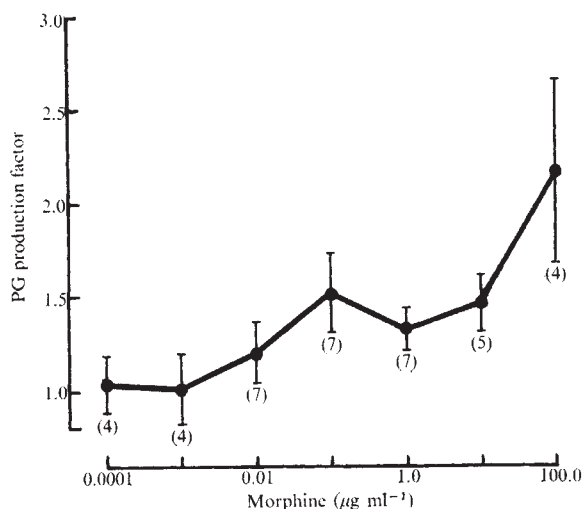


Fig. 2 Stimulation by morphine of the PG synthetase of bull seminal vesicles. Values are the means of 4–7 independent experiments. Other details as in Fig. 1.

whether prostaglandins can elicit vomiting or hyperglycaemia also by a central action. We therefore propose that the hyperthermic effects of apomorphine and morphine, and possibly also their emetic, hyperglycaemic and allied effects, may be due to the stimulation of prostaglandin biosynthesis. This proposition requires that chlorpromazine, which inhibits apomorphine-induced vomiting in the dog^{23,24}, should inhibit the stimulation by apomorphine of PG synthetase; and we have found this to be so.

It has recently been proposed that morphine may exert its analgesic or allied effects by inhibiting the stimulation by E prostaglandin of cyclic AMP formation²⁵. Thus, diverse interactions with prostaglandin mechanisms might explain some depressant and excitatory effects of morphine on the nervous system and provide the basis for a mechanism of tolerance/dependence.

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Inhibition of glycoprotein and glycolipid synthesis in hamster embryo cells by cytosine arabinoside and hydroxyurea

THE nucleoside, 1-β-D-arabinofuranosylcytosine (ara-C) and the unrelated compound, hydroxyurea, are known to inhibit DNA replication in bacterial and animal cells^{1–3}. Arabinofuranosyl cytidine-5'-triphosphate (ara-CTP) specifically interferes with the action of DNA polymerase II in bacterial cell extracts⁴, whereas hydroxyurea affects the ribonucleoside diphosphate reductase reaction⁵. We have shown that ara-C and hydroxyurea bring about transformation of hamster embryo cells in tissue culture⁶. Further, it has also been demonstrated that ara-C inhibits the incorporation of ³H-D-glucosamine (a precursor of N-acetylneuraminic acid) into glycoproteins and glycolipids of hamster embryo fibroblasts and transformed cells⁷. In continuing these experiments we have been interested in, first, determining the site or sites of action of ara-C (presumably as its triphosphate) in the cell and second, studying other inhibitors of DNA replication as to their possible effect on glycoprotein and glycolipid synthesis.

To compare the effect of ara-C and other chemicals on both DNA replication and glycoprotein/glycolipid synthesis, either secondary cultures of hamster embryo fibroblasts or transformed hamster embryo cells were used as the test system⁶. Cells were exposed to the different chemicals for 24 h, and the incorporation of ¹⁴C-thymidine and ³H-D-glucosamine measured into acid-precipitable material at selected times during this period.

As expected, ara-C and hydroxyurea are potent inhibitors of DNA replication, while FUDR and the carcinogenic hydrocarbons were less effective in this respect (Table 1). With regard to the incorporation of ³H-D-glucosamine (glycoproteins and glycolipids), ara-C inhibited by 86% in normal cells and 50% in transformed cells (Table 1). Higher inhibitions have been observed with transformed cells in further experiments. Hydroxyurea brought about a 70% inhibition of incorporation in normal cells and a 75% inhibition in transformed cells, FUDR and the polycyclic hydrocarbons inhibited incorporation but to a much lesser extent (20–30%). Figure 1 shows the time curve of incorporation of ¹⁴C-thymidine (Fig. 1a) and ³H-D-glucosamine (Fig. 1b) into DNA and glycoproteins/glycolipids of trans-

Table 1 The effect of ara-C, hydroxyurea and other chemicals on the incorporation of ^{14}C -thymidine into DNA and ^3H -D-glucosamine into glycoproteins/glycolipids of cells in tissue culture

System	Incorporation ^{14}C -thymidine (c.p.m./mg protein $\times 10^{-3}$)		% Inhibition		Incorporation ^3H -glucosamine (c.p.m./mg protein $\times 10^{-3}$)		% Inhibition	
	Transformed	Normal	Transformed	Normal	Transformed	Normal	Transformed	Normal
Control	388	567	—	—	200	122	—	—
Ara-C (10^{-3} M)	11.4	89.7	97%	84%	100	17.2	50%	86%
Hydroxyurea (10^{-2} M)	4.9	8.2	99%	99%	49	25.4	75%	79%
FUdR (10^{-3} M)	235	377	39%	43%	138	89	31%	27%
Methylcholanthrene ($10 \mu\text{g ml}^{-1}$)	277	495	28%	13%	157	107	22%	21%
Benzpyrene ($10 \mu\text{g ml}^{-1}$)	267	461	31%	19%	138	106.5	31%	23%

Experimental details as for Fig. 1. Incubations were at 37°C for 24 h. Four other individual experiments gave similar results.

formed cells and the effect of ara-C and hydroxyurea on these reactions. The results indicate continuous inhibition of incorporation of both radioactive precursors over 24 h.

The observations recorded in Table 1 and Fig. 1 on the simultaneous inhibition of DNA replication and glycoprotein/glycolipid synthesis by ara-C and hydroxyurea in particular, suggest some correlation between these two synthetic pathways. It is worth mentioning that ara-C (10^{-3} M) inhibits ^3H -uridine incorporation by 3% (normal cells) and 11% (transformed cells) after 24 h of incubation. Protein synthesis was stimulated 3% (normal cells) and inhibited by 7% (transformed cells) at the same time period. On the other hand, hydroxyurea (10^{-2} M) inhibited ^3H -uridine incorporation by 30% (normal cells) and 17% (transformed cells) after 24 h of incubation. Protein synthesis was inhibited by 12% (normal cells) and 3% (transformed cells) respectively at the same time period. Ara-C

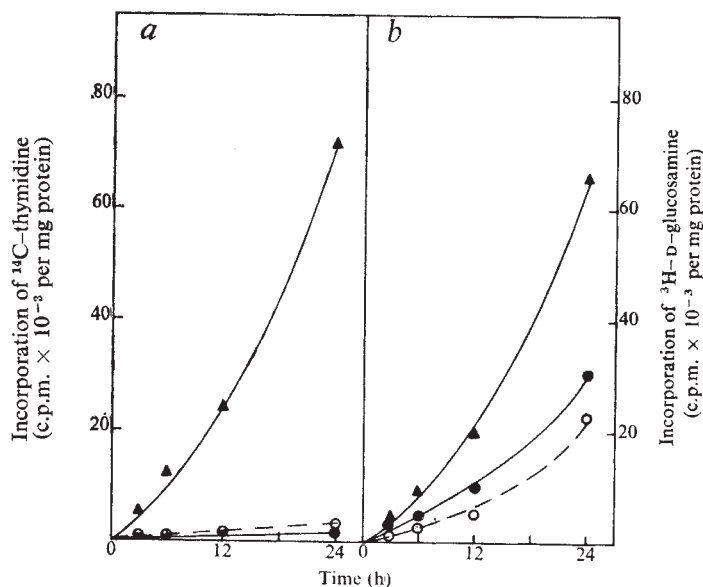


Fig. 1 Effect of ara-C and hydroxyurea on the incorporation of ^{14}C -thymidine into DNA and ^3H -D-glucosamine into glycoproteins and glycolipids of transformed hamster embryo cells. Incubations were carried out in Falcon Petri dishes (5.5 cm diameter) containing 5.0×10^5 cells in 3 ml of growth medium (Eagle's Minimal Essential medium containing Earle's salts, 10% calf serum, penicillin and streptomycin) at 37°C in a 5% CO_2 atmosphere. Incorporation experiments were commenced after approximately 24 h, by which time the cells had half mono layered. Ara-C was present at a concentration of 10^{-3} M and hydroxyurea at 10^{-2} M. Each Petri also contained $1.0 \mu\text{Ci}$ ^{14}C -thymidine and $0.5 \mu\text{Ci}$ ^3H -D-glucosamine per ml incubation medium. At selected times, the medium was removed, the cells rinsed with PBS, and the cell pellet homogenised in 1 ml of water by means of a syringe using a No. 21 needle. Aliquots were taken for counting and protein determination¹³. For counting, samples were precipitated with cold 10% trichloroacetic acid (TCA), transferred to Whatman GF/C filters and washed with 25 ml of cold 5% TCA. Counting was carried out in a Scintillation Spectrometer. *a*, ^{14}C -thymidine incorporation. $\blacktriangle$, control; $\circ$, ara-C; $\bullet$, hydroxyures. *b*, ^3H -D-glucosamine incorporation. $\blacktriangle$, control; $\circ$, ara-C; $\bullet$, hydroxyurea.

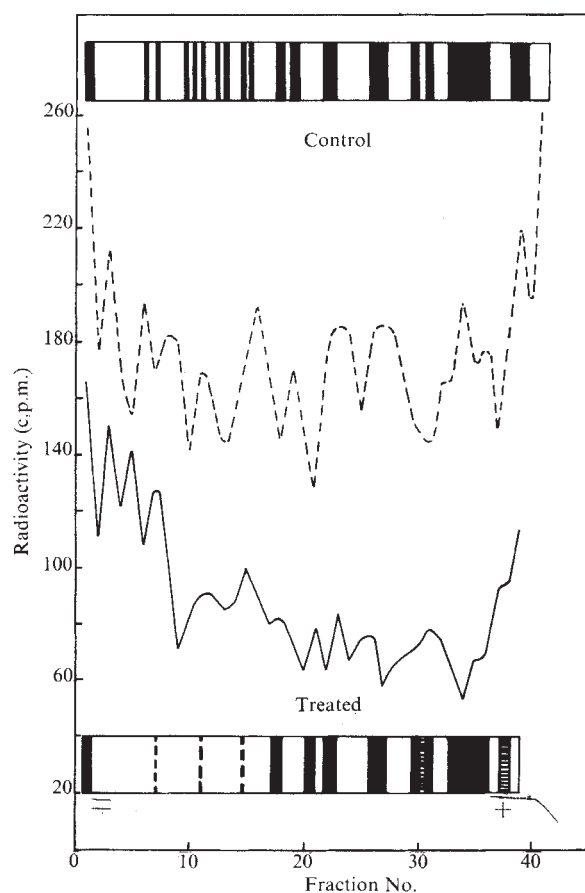


Fig. 2 Polyacrylamide gel electrophoresis of cell membrane glycoproteins. Cells were grown in Roux Flasks as described for Fig. 1. ^3H -D-glucosamine was present at $1.0 \mu\text{Ci}$ per ml incubation medium. Ara-C when present was at a concentration of 10^{-3} M. After 24 h at 37°C , cells were washed with PBS and removed from the glass by treatment with PBS containing 0.02% (w/v) EDTA for 30 min at 37°C . Cell membranes were isolated by a modified method based upon the procedures of Neville¹⁴ and Graham¹⁵. Glucose-6-phosphatase and 5'-nucleotide activities were assayed according to the methods of Swanson¹⁶ and Heppel and Hilmire¹⁷, respectively. Polyacrylamide gel electrophoresis was carried out in buffers according to Jones¹⁸. Protein was stained with Amido Black and gel slices were treated with H_2O_2 before counting in Bray's solution. ---, Radioactivity in glycoproteins of control cells; —, radioactivity in glycoproteins of cells treated with 10^{-3} M ara-C.

has also been found to have no effect on the incorporation of CTP into the 3'-terminus of tRNA catalysed by the nucleotide-incorporating enzyme. The latter experiments were carried out *in vitro* with rat liver enzymes and chemically synthesised ara-CTP (T.S-B., and L. Parkin, unpublished). Further, as previously reported⁷, ara-C does not affect the incorporation of ^{14}C -choline into phospholipids of normal or transformed hamster embryo fibroblasts.

We compared the patterns of labelling of glycoproteins of the cell membranes of both normal and ara-C treated cells

Table 2 The effect of ara-C and hydroxyurea on the transfer of sugars from their respective nucleotide carriers to glycoproteins of the cell membrane

System	Incorporation into TCA precipitable material (c.p.m./mg protein)		% Inhibition
	Control	Plus 10^{-3} M ara-C	
14 C-UDP-galactose	1,509	730	52
3 H-UDP-glucose	1,205	1,220	—
14 C-UDP-N-Ac-glucosamine	775	216	73
14 C-CMP-sialic acid	523	160	69
		Plus 10^{-2} M hydroxyurea	
14 C-UDP-galactose	1,250	620	50
3 H-UDP-glucose	4,950	5,050	—
14 C-UDP-N-Ac-glucosamine	580	240	59
14 C-CMP-sialic acid	740	220	70

Transformed cells were pre-incubated in Petri dishes until approximately half monolayered as described for Fig. 1. After removal of the medium, cells were rinsed with 0.01 M Tris-HCl (pH 7.6) + 0.15 M NaCl and then incubated in a buffer containing sucrose (0.15 M); Tris-HCl, pH 7.6 (40 mM); KCl (15 mM); $MgCl_2$ (6.6 mM) and $MnCl_2$ (6.6 mM). Radioactive nucleotide sugars were present at 1 μ Ci per 3.0 ml incubation medium. Incubation was for 1 h at 37° C. Cells were processed for radioactivity and protein content as described for Fig. 1.

For this purpose, cells with ara-C (10^{-3} M) and without ara-C were incubated for 24 h with 3 H-D-glucosamine. Thereafter, cell membranes were isolated and washed prior to analysis by means of polyacrylamide gel electrophoresis. The results of these experiments are shown in Fig. 2. Definite differences are seen as regards the distribution of proteins between the normal and ara-C-treated cells. Also, the labelling of the glycoproteins is generally much lower in the ara-C-treated cells as compared with the control cells. Similar results have been obtained with the glycoproteins from cells treated with hydroxyurea.

Various research workers have shown that labelled sugars can be transferred from their activated nucleotide carriers to receptors on the cell surface⁸⁻¹⁸. It is assumed that cell surface glycosyl transferases carry out these reactions. It was of considerable interest to ascertain whether ara-C and hydroxyurea were capable of interfering with these transferase reactions at the cell surface or not. The transfer of labelled D-galactose, D-glucose, N-acetyl D-glucosamine and sialic acid from their respective nucleotide carriers to cell surface glycoproteins was carried out as described in Table 2. It can be seen that ara-C and hydroxyurea inhibit the transfer of galactose, N-acetyl D-glucosamine and sialic acid to a considerable extent, whereas the transfer of glucose does not seem to be affected.

The inhibitions observed could possibly be due to direct interaction of the inhibitors with the glycosyl transferase enzymes of the cell surface (see Table 2). Preliminary experiments with isolated microsomes from transformed hamster embryo cells and rat liver have shown that ara-C (10^{-3} M) has no effect on the transfer of labelled sugars from their respective nucleotide carriers to endogenous receptors present in the microsomes. On the other hand, ara-CTP (10^{-3} M) strongly inhibits the transfer of sugars from their carriers to microsomes. The transfer of UDP-galactose was inhibited by approximately 80%, UDP-N-acetyl-glucosamine and GDP-mannose both by 60% and CMP-sialic acid by 25%. Hydroxyurea (10^{-2} M) also inhibited direct transfer of the sugars but to a lesser extent. These preliminary results suggest a direct interference with the glycosyl transferases by the inhibitors discussed. They also suggest that the inhibition of glycoprotein/glycolipid synthesis observed is not due to indirect effects operating through the inhibition of DNA replication.

In the case of ara-C, where presumably the effective inhibitor is ara-CTP, inhibition occurs through direct interaction with the enzyme activating N-acetylneuraminic acid (NANA). Work in our laboratory (L. Parkin and A.O.H., unpublished) with chemically synthesised ara-CTP has shown that the nucleotide inhibits activation in rat liver nuclear extracts¹¹ and with the partially purified enzyme from hog submaxillary glands¹². Maximum inhibition has been of the order of 50–60%. We have also shown pre-

viously⁷ that ara-CMP (10^{-3} – 10^{-5} M) inhibits the transfer of NANA from CMP-NANA to receptors by approximately 20%. It seems therefore that ara-C as its triphosphate is inhibiting both the activation and subsequent transfer of NANA to receptors. The effect of ara-CTP on the transfer of other activated sugars to receptors in isolated microsomes is of considerable interest. These are direct effects and are under study at the moment.

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Assignment of the human gene for galactokinase to chromosome 17

EVIDENCE presented here and in the report by Elsevier *et al.*¹ mutually establishes the assignment of the human gene for galactokinase (EC 2.7.1.6), the enzyme which converts galactose to galactose-1-phosphate, to chromosome 17. This assignment is based on a syntenic relationship between the genes for thymidine kinase and galactokinase.

Hybridisation of rodent and human somatic cells and the preferential loss of human chromosomes which segregate in the

resultant hybrid clones has become an important procedure for assigning human genes to specific chromosomes². When the HAT selection system³ is used to select hybrids produced by fusion of a thymidine kinase-deficient rodent cell line and a thymidine kinase-positive human cell line, the human gene for thymidine kinase is retained since activity of this enzyme is required for cell survival in HAT medium⁴. Using this selection system the gene for human thymidine kinase was first assigned to a group E chromosome^{5,6}; later specifically to chromosome 17 (ref. 7), and more recently to the long arm of this chromosome⁸. When HAT selected hybrids are placed in medium containing 5-bromodeoxyuridine (BrdU), the incorporation of this thymidine analogue into DNA, made possible by thymidine kinase activity, is lethal, thereby producing selection against cells retaining the thymidine kinase gene⁹. Furthermore, the hybrid cells which can grow in medium containing BrdU, have lost the human chromosome 17 (ref. 9).

The hybrid clones analysed for human galactokinase activity were derived independently from five separate fusion experiments involving mouse (Cl1D) and human (KOP-2, LNSV and W18Va2) parental cell lines. These parental cell lines and the procedures used for cell fusion have been described before⁹. After fusion, hybrid cells were maintained in HAT selective medium and subsequently cloned. Each clone was derived from a different hybrid cell colony. Nine of the HAT selected hybrid cell clones were transferred to medium containing $30 \mu\text{g ml}^{-1}$ BrdU and maintained in it for at least 30 passages. Mouse and human chromosomes were identified in each of the hybrid cell clones by Giemsa-banding as previously described^{9,10}. A minimum of 20 metaphases from each hybrid cell clone was analysed to identify the human chromosomes present.

Thirty-two human-mouse hybrid cell clones were tested for the presence of human galactokinase. Extracts of these hybrid clones were electrophoresed in starch by the methods of Tedesco *et al.*¹⁰. In this procedure, enzyme activity is localised by staining the gel with a reaction mixture^{10,11} containing 0.5% Ionagar No. 2 (Consolidated Laboratories Inc.) 0.2 M Tris-HCl, pH 8.0, 7 mM β -mercaptoethanol, 2 mM MgCl_2 , 0.94 mM NADP, 0.6 mM UDP-glucose, 0.21 mM glucose-1, 6-diphosphate, 0.4 mM galactose, 6.6 mM ATP, 0.1 U ml^{-1} yeast galactose-1-P uridyl-transferase (Sigma), 0.2 mg phosphoglucomutase (Boehringer-Mannheim), 0.2 mg glucose-6-phosphate-dehydrogenase (Boehringer-Mannheim), and 0.2 mg

Table 1 Syntenic relationship between human galactokinase and thymidine kinase

	Human Gk +	Human Gk -
HAT selected clones	23	0
BrdU selected clones	0	9

6-phosphogluconate-dehydrogenase (Boehringer-Mannheim). As a final step in the reaction series, NADPH is produced which can be visualised under long wave ultraviolet light. This electrophoretic system easily separates human galactokinase from mouse enzyme, as Fig. 1 shows.

All 23 hybrid clones selected in HAT medium contained the human chromosome 17 and displayed human galactokinase activity (Table 1). On the contrary, nine clones which were back-selected in medium containing BrdU and which have lost human chromosome 17, did not contain human galactokinase activity (Fig. 1 and Table 1).

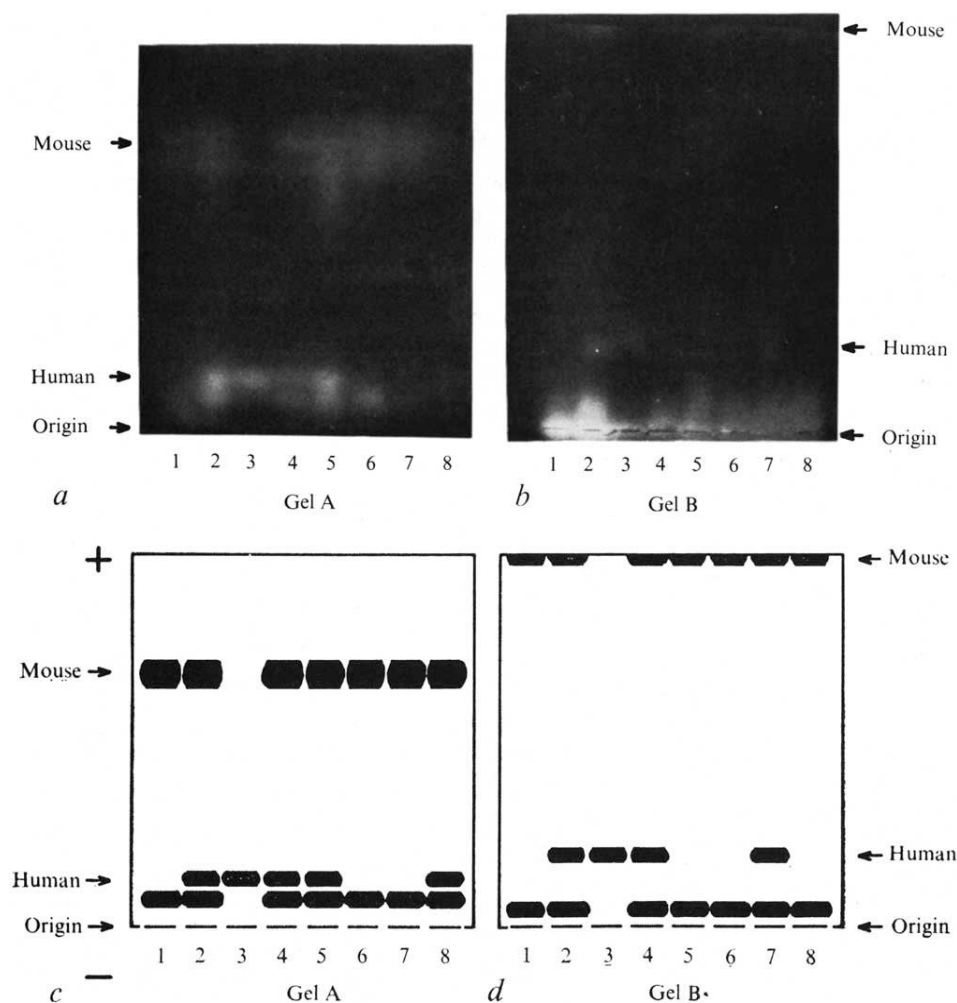


Fig. 1 Two different starch gels stained for galactokinase activity as before¹⁰, photographed under long wave ultraviolet light (a and b), c and d are diagrammatic representations of a and b respectively. Galactokinase extracted from mouse (Cl1D) and human (LNSV) parental cells each migrate as a single band of enzyme activity and are well separated in this electrophoretic system. Gel A was run at 8 V cm^{-1} and gel B at 10 V cm^{-1} for 18 h at 4°C . Gel A demonstrates the wide degree of separation between human and mouse enzyme obtained in this system. All the hybrid clones tested were run with the higher voltage to obtain more effective separation of human galactokinase from the nonspecific staining that occurs near the origin in these gels when galactose is deleted from the reaction mixture. Under these conditions mouse galactokinase migrates into the anode wick area of the gel. Gel A: channel 1, 52-58 clone 8 BrdU; 2, 52-58 clone 8; 3, LNSV (human parental cell line); 4, 52-58 clone 9; 5, 52-67 clone 21; 6, 52-67 clone 21 BrdU; 7, Cl1D (mouse parental cell line); 8, 52-58 clone 19. Gel B: channel 1, 52-58 clone 8 BrdU; 2, 52-58 clone 8; 3, LNSV; 4, 52-58 clone 9; 5, 52-58 clone 3 BrdU; 6, Cl1D; 7, 52-67 clone 21; 8, 52-67 clone 21 BrdU. In gel A channels 1 and 2 and 5 and 6, and in gel B channels 1 and 2 and 7 and 8 represent pairs of HAT selected clones before and after BrdU selection.

These data demonstrate the syntenic relationship between human galactokinase and thymidine kinase and allow the assignment of the human gene for galactokinase to chromosome 17.

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Opossum Hb chain sequence and neutral mutation theory

THE divergence of amino acid sequences which has accompanied evolution of species may be largely due to random fixation of selectively neutral or nearly neutral mutations. This was proposed by Kimura upon consideration of an inferred rapid rate of molecular evolution, the theory of the cost of natural selection, and observed high levels of polymorphism in natural populations¹. Among the observations considered to favour the neutral mutation-random fixation theory¹⁻⁴, the apparent uniformity of the rate of fixation of amino acid substitutions in homologous proteins along several phyletic lines is outstanding. Kimura proposed this test of the neutral mutation theory by examining the amino acid sequences of proteins from so-called 'living fossils'⁴. If, for a very long slowly evolving line, a rate of molecular evolution equal to that of more rapidly evolving lines is inferred (and it is assumed that the rate of evolution of physiological adaptations generally parallels the morphological evolutionary rate) then a case is made for the fixation of neutral mutations. The amino acid sequence proposed here⁵ (Fig. 1) for the α chain of haemoglobin from a 'living fossil', *Didelphis marsupialis*, the Virginia opossum may be taken for such a test of the neutral mutation theory.

Since only a single haemoglobin component was found by gel electrophoresis⁷, chain separation⁸ was done on globin prepared from fresh haemolysate. Cyanogen bromide treatment⁹ of the α chain yielded fragments containing residues 1-25, 26-76, 110-125, and 126-141; treatment with citraconic anhydride¹⁰, ethyleneimine¹¹, and trypsin gave frag-

```

1                                     15
Val-Leu-Ser-Ala-Asn-Asp-Lys-Thr-Asn-Val-Lys-Gly-Ala-Tyr-Ser-
                                     30
Lys-Val-Gly-Gly-Asn-Ser-Gly-Ala-Tyr-Met-Gly-Glu-Ala-Leu-Tyr-
                                     45
Arg-Thr-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-Asn-
                                     60
Tyr-Asp-Phe-Ser-Ala-Gly-Ser-Ala-Gln-Ile-Lys-Thr-Gln-Gly-Gln-
                                     75
Lys-Ile-Ala-Asp-Ala-Val-Gly-Leu-Ala-Val-Ala-His-Leu-Asp-Asp-
                                     90
Met-Pro-Thr-Ala-Leu-Ser-Ser-Leu-Ser-Asp-Leu-His-Ala-His-Glu-
                                     105
Leu-Lys-Val-Asp-Pro-Val-Asn-Phe-Lys-Phe-Leu-Cys-His-Asn-Val-
                                     120
Leu-Val-Thr-Met-Ala-Ala-His-Leu-Gly-Lys-Asp-Phe-Thr-Pro-Glu-
                                     135
Ile-His-Ala-Ser-Met-Asp-Lys-Phe-Leu-Ala-Ser-Val-Ser-Thr-Val-
                                     141
Leu-Thr-Ser-Lys-Tyr-Arg

```

Fig. 1 Proposed amino acid sequence for *Didelphis marsupialis* haemoglobin α chain.

ments containing residues 1-31, 32-102, and 103-141. Fragments were identified by compositions and sequences of tryptic peptides derived from them: identification of each tryptic peptide was generally inferred from obvious homology with α chains of known sequence. Identification of the peptide containing residues 57-61 was made by compositions of the parent fragments and identities of the other tryptic peptides derived from those fragments. Fragments and peptides were purified with columns of Sephadex or cation-exchange resins¹². Sequence determination of the tryptic peptides was done by further enzymic or chemical cleavage and the subtractive Edman technique¹³. Amides were determined by aminopeptidase M digestion¹⁴ or glycinamidation¹⁵. (Full details of the structure determination will be published elsewhere.)

In Fig. 2 the number of sequence differences between members of several pairs of vertebrate α chains¹⁶⁻²⁴ are listed. The opossum α chain has apparently evolved more rapidly than any other α chain in all cases in which a comparison is possible. This result exceeds the expectations of the neutral mutation theory. A selectionist interpretation of the more rapid rate of molecular evolution in a living fossil is possible: if the evolutionary rate is limited by the cost of natural selection²⁵⁻²⁷ then one may expect to find an

	Cow	Horse	Dog	Rabbit	Man	Kangaroo	Opossum	Echidna	Chicken	Carp
Mammalia										
Eutheria										
Cow ¹⁶	0	18	28	25	17	26	43	43	38	65
Horse ¹⁷		0	27	25	18	29	42	42	40	67
Dog ¹⁸			0	27	23	33	46	42	44	67
Rabbit ¹⁹				0	25	37	50	48	43	71
Man ²⁰					0	27	40	37	35	68
Metatheria										
Kangaroo ²¹						0	42	49	41	71
Opossum							0	60	60	77
Prototheria										
Echidna ²²								0	48	75
Aves										
Chicken ²³									0	72
Osteichthyes										
Carp ²⁴										0

Fig. 2 Amino acid sequence differences between several pairs of vertebrate haemoglobin α chains. Comparisons at sites involving the relative insertions or deletions in the carp sequence were not included.

especially keen rate of molecular evolution accompanying slow morphological changes because the reduced number of loci undergoing substitutions may sustain more intense selection per locus.

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Preservation of antigenic properties of macromolecules over 70 Myr

It has been known for some time that many fossils still contain remnants of organic macromolecules, such as proteins and polysaccharides¹. Such macromolecules are often enclosed inside the mineralised tissues of fossils, well protected from outside influences. This close association of skeletal, mineral and macromolecular substances, which is also found in recent mineralised tissues, suggests that this organic material has been an important factor in the process of mineralisation and has deeply influenced the physical properties of the skeletons^{2,3}. It cannot be excluded however, that organic substances not involved in mineralisation can also be preserved in sedimentary rocks without much loss of their original structure, provided that they are well enclosed in the sediment. With this reserva-

tion in mind, characterisation of the structure of enclosed substances may yield valuable palaeobiochemical and diagenetical information.

Fossil macromolecules are often very complex, may occur in a great variety and may contain multiple decomposition products. Chemical analyses have hitherto been confined mainly to qualitative and quantitative determinations, such as amino acid analyses. In this paper we show that immunological techniques are very useful for the comparison of fossil and recent material from different sources. They may also be of value for the study of diagenetical processes.

We investigated cephalopods because fossil as well as recent representatives are readily available and contamination can easily be eliminated. Shells of the recent *Sepia officinalis* from the Dutch coast, of *Nautilus pompilius* (recent—Indian Ocean) and of *Belemnitella junior* (Maastrichtian—Maastricht, Netherlands) were scrupulously cleaned from organic and inorganic contamination and ground to a fine powder. Each powder was then decalcified in excess of 10% EDTA solution (pH 8) and insoluble residues were removed by centrifugation (30 min at 20,000g). The supernatant was concentrated and dialysed with an Amicon PM 10 filter until free of EDTA. One ml aliquots containing 1-2 mg material from *Sepia* and *Belemnitella* were injected with complete Freund's adjuvant into rabbits three times subcutaneously at intervals of two weeks. Against each antigen two antisera were prepared (AS_I and AS_{II} against *Sepia* and AB_I and AB_{II} against *Belemnitella*). Extracts from the three different sources were compared in the Ouchterlony technique⁴ (Fig. 1). When the three extracts were allowed to react with preimmunisation sera, no precipitates were found. All the precipitates described are therefore immunoprecipitates.

Both *Sepia* antisera reacted with *Sepia*, *Belemnitella* and *Nautilus* extracts (Figs 1a and b). Depending on the antigen preparation (S_I or S_{II}) used, one or two precipitation lines were formed (Figs 1a and b). The AS_{II} serum indicates cross-reactivity between *Sepia* and *Belemnitella* antigens, which is not shown with the AS_I serum (Figs 1a and c). The AS_{II} serum also detects crossreactivity between the *Nautilus* and *Belemnitella* antigens (Fig. 1d). Both AS sera produce very sharp lines with the *Nautilus* extract (Figs 1b and e). When tested with the AS_I serum, no crossreactivity between the *Sepia* and *Nautilus* antigens is seen (Fig. 1e). This may be due to either the absence of the crossreacting antigen from the S_I antigen preparation (Figs 1a and b) or a crossreactivity between the *Nautilus* antigen and a hidden determinant of the *Sepia* antigens.

The experiments with the anti-*Belemnitella* sera produced only weakly visible patterns. From Fig. 1f it is clear, however, that single precipitation lines are formed with *Belemnitella* and *Nautilus* extracts indicating complete crossreactivity. The *Sepia* extract does not react with anti-*Belemnitella* serum, although the *Belemnitella* extract does react with the anti-*Sepia* sera (Figs 1a and c). This may indicate a weaker immunogenicity of the *Belemnitella* extract. From these experiments the following conclusions can be drawn. First, fossil as well as recent calcified tissues have been found to contain organic material with antigenic properties; second, because certain fractions from *Belemnitella* still crossreact with extracts of shells from recent *Sepia* (partially) identical structures have been retained in the fossil macromolecules for over 70 Myr. Third, the reactions of the *Nautilus* extract with the anti-*Sepia* and anti-*Belemnitella* sera suggest that the skeletal macromolecules in question have undergone minor alterations since the lower Carboniferous, when the Coleoids are thought to have diverged from the Nautiloid lineage. In evolutionary terms these must have been extremely conservative substances.

The antigenic relationship of the skeletal macromolecules of Cephalopods and related taxa is now being worked out in detail in our laboratory. The possibility of antigen localisation in sedimentary rocks by immunological techniques at light-

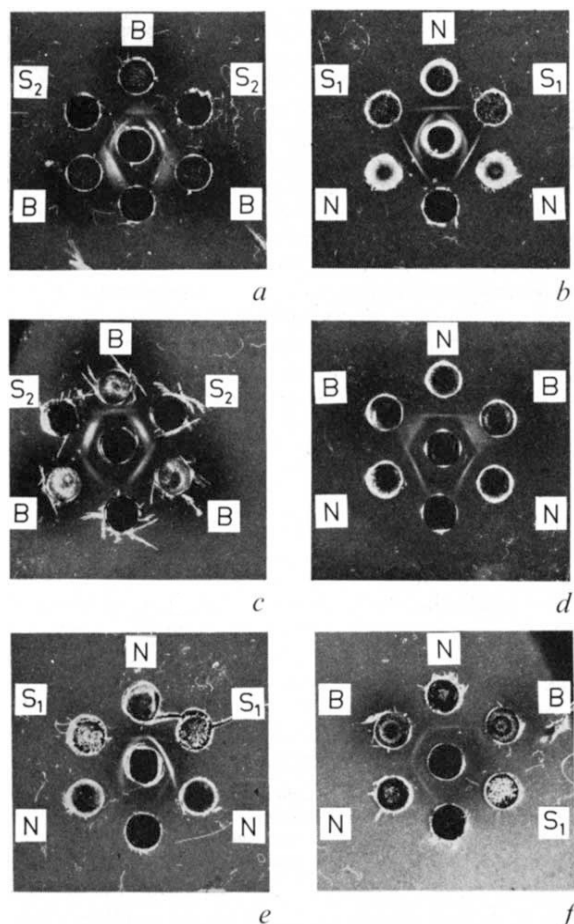


Fig. 1 Immunodiffusion patterns of extracts from shells of *Sepia officinalis*, *Nautilus pompilius* (both recent) and 70-Myr-old *Belemnitella junior*. The central wells contained: anti-*Sepia* serum (AS_1) (c and e); anti-*Sepia* serum (AS_{11}) (a, b and d); Anti-*Belemnitella* serum (AB_{11}) (f). B; *Belemnitella* extract; S_1 and S_2 , *Sepia* extracts; N, *Nautilus* extract.

and electron-microscopical level is also being investigated. Determination with immunological techniques of the phylogenetic relationship of taxa with unknown systematic position, such as the stromatoporoids, is yet another project which we intend to investigate in the near future.

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Defective membrane synthesis in an *E. coli* mutant

THERE is a strict correlation between DNA synthesis and cell division^{1,2} but there is no known relationship between cell division and membrane synthesis—presumably because so little is known about the latter. We have previously reported that the formation of cytochrome b_1 and the L- α -glycerophosphate uptake system occurs at the same time (in a discontinuous manner) at a particular phase in a cell cycle of *Escherichia coli* (ref. 3). On the basis of these observations we attempted to isolate a phase-specific mutant which has a defect in the formation of membrane components. The selection of the mutants was carried out assuming that cell growth is temperature-sensitive and that the formation of the β -galactoside transport system is under the control of the corresponding *ts*-mutation. These assumptions were based on the observation that the formation of the transport system for β -galactoside, measured using a synchronous culture, completely coincided with those of many membrane components including cytochrome b_1 (M.O., unpublished) and on the consideration that cells defective for such processes should be non-viable⁴.

Here we present evidence that the mutant we isolated is unable to form cytochrome b_1 , an L- α -glycerophosphate transport system, an L- α -glycerophosphate dehydrogenase and succinate dehydrogenase at a non-permissive temperature, because of a single mutational defect in a phase-specific function which operates 20-30 min before cell division. We also postulate two alternative functions of a protein which is synthesised or activated at 20-30 min before cell division. First, that the protein controls the fabrication of the active forms of membrane components, and, second, that the protein regulates a concurrent expression of the genes responsible for cytochrome b_1 and so on, thus resulting in alignment of a population at a specific phase of cell division at a non-permissive temperature.

The mutant *tsC42*, described here, grows at a normal rate at 30°C but it stops growing at 42°C. Figure 1 shows that protein synthesis takes place at 42°C. The increase in the amount of protein began rapidly but gradually stopped. Finally, it reached a level of 1.7-1.8 times the original amount. The turbidity of the culture, the amount of protein and viable

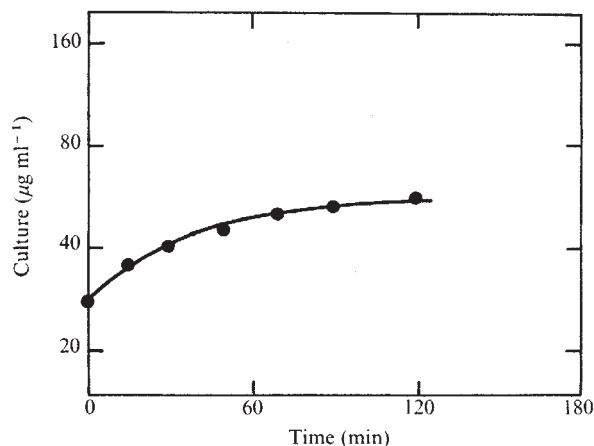


Fig. 1 Synthesis of protein in the mutant *tsC42*. Cells were grown in A medium⁵ for several generations at 30°C and transferred to 42°C. Samples were withdrawn at the times indicated. Protein was measured by the method of Lowry *et al.*⁶.

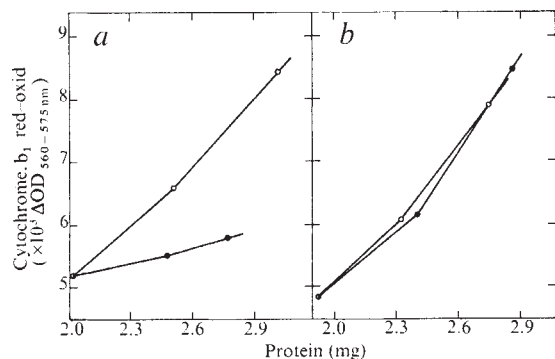


Fig. 2 Formation of cytochrome b_1 in the mutant *tsC42* and the parental strain AT713. Cells were grown in A medium⁵ for more than three generations at 30° C and the culture divided into two parts. One portion was heated to 42° C and the other was further grown at the same temperature. Samples were removed after 20 and 40 min in the incubation at 42° C (●) and 30 and 60 min in the incubation at 30° C (○). The amount of cytochrome b_1 was determined as described previously³. *a*, *tsC42*; *b*, AT713.

cells remained constant for a long period of incubation after initial increases. The size of the cells accumulated during incubation at a non-permissive temperature were homogeneous and almost equivalent to the larger cells of a cell population growing randomly.

Cytochrome b_1 is the major component of cytochromes in *E. coli* and a membrane-bound constituent which is constitutively synthesised³. Figure 2 shows the results of experiments in which the formation of cytochrome b_1 was measured in the mutant *tsC42* and the parent AT713. The rate of formation was greatly reduced at 42° C, compared with 30° C in the mutant *tsC42* (Fig. 2*a*), while their rates were essentially identical in the parent AT713 (Fig. 2*b*).

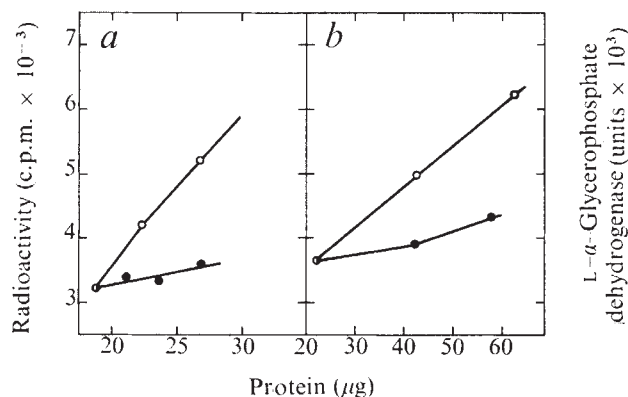


Fig. 3 Formation of L- α -glycerophosphate transport and L- α -glycerophosphate dehydrogenase in the mutant *tsC42*. Cells were grown in A medium⁵ supplemented with 0.04 M DL- α -glycerophosphate. The assay of ^{14}C -L- α -glycerophosphate uptake in samples withdrawn after 30 and 60 min incubation at 30° C (○), and 20, 40 and 60 min at 42° C (●), was carried out according to the method of Hayashi *et al.*⁷. For the assay of L- α -glycerophosphate dehydrogenase, samples were withdrawn after 30 and 60 min incubation at 30° C (○) and 20 and 40 min incubation at 42° C (●) and the cells were washed twice with 0.1 M phosphate (pH 7.4) at 0° C and suspended in the same buffer. The cells were disrupted by treatment in a Umeda 20 kc 150 W sonic oscillator for 1 min and the unbroken cells were removed by centrifugation at a low speed. The enzymatic activity was assayed by measuring the rate of reduction of DCIP (2,6-dichlorophenol-indophenol) essentially according to the procedure of Lin *et al.*⁸ except for the use of DCIP instead of MTT and measuring at 600 nm. The assay mixture contained: 0.30 ml cell-free extract, 2.0 ml 0.1 M phosphate at pH 7.4, 0.06 ml DCIP (1 mg ml⁻¹), 0.30 ml phenazine methosulphate (1 mg ml⁻¹), 0.20 ml 0.15 M KCN and 0.25 ml 0.4 M DL- α -glycerophosphate. One unit corresponds to 1 μmol DCIP reduced min⁻¹. *a*, Transport of ^{14}C -L- α -glycerophosphate; *b*, L- α -glycerophosphate dehydrogenase.

Figure 3*a* shows the inability to form the transport system for L- α -glycerophosphate when the culture of the mutant was transferred into 42° C (after a full induction of the system by the supplement of 0.04 M DL- α -glycerophosphate). Since the transport once formed at a permissive temperature was fully active at 42° C, and as both the activities at 30° C and 42° C and the ratio of 42°:30° C are almost identical with those of the parental strain AT713, the inability of the inducer transport is not a cause for the absence of formation of the system at a non-permissive temperature. The inability of the mutant to form membrane components is also demonstrated for other enzymes. The results shown in Fig. 3*b* and 4 are for inducible membrane-bound L- α -glycerophosphate dehydrogenase and succinate dehydrogenase, being a typical and a constitutive membrane enzyme, respectively.

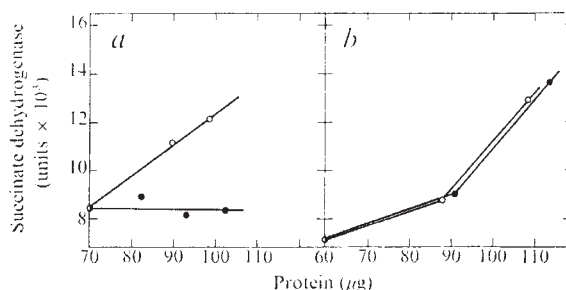


Fig. 4 Formation of succinate dehydrogenase in the mutant *tsC42* and the parent AT713. The cultivation of cells and the preparation of the enzyme fraction are the same as described in the legend to Fig. 3 except that the induction by L- α -glycerophosphate was omitted. The enzymatic activity was measured essentially according to the method described in the legend to Fig. 3. The assay mixture contained: 0.30 ml cell sonicate, 1.60 ml 0.1 M phosphate at pH 7.4, 0.06 ml DCIP (1 mg ml⁻¹), 0.30 ml phenazine methosulphate (1 mg ml⁻¹), 0.20 ml of 0.15 M KCN and 0.60 ml 0.5 M succinate. A unit corresponds to μmol of DCIP reduced min⁻¹. *a*, *tsC42*; *b*, AT713.

The spontaneous reversion frequency to resistant cells at 42° C in *tsC42* is between 10^{-6} to 10^{-7} ; within that known for single mutations. All three revertants examined were capable of forming cytochrome b_1 , succinate dehydrogenase and L- α -glycerophosphate dehydrogenase at equal rates (increase of activity/increase of protein) at both 30° and 42° C, indicating that the pleiotrophic defect observed in the formation of several membrane components and cell growth was caused by a single mutation.

Figure 5 shows the patterns of cell division and the formation of succinate dehydrogenase when cells were transferred to 30° C after 35 min incubation at a restrictive temperature (42° C). Cell division occurred synchronously at 35 min and 135 min after a temperature shift to 30° C. The age of cells accumulated during the incubation at 42° C, deduced from the pattern of cell division, corresponds to 0.65 of a generation (designated as zero or one for cells at mid phase of cell division) and is compatible with the observed ages when many membrane components such as cytochrome b_1 were formed during a synchronous growth⁹. The formation of succinate dehydrogenase occurred immediately after the shift from a non-permissive to a permissive temperature in a discontinuous manner. This result is consistent with the view that because of a defect in the regulation of membrane components formation the whole population of cells aligned at a phase 20–30 min before cell division during an incubation at a non-permissive temperature. Furthermore, similar immediate formation was also observed in the case of the L- α -glycerophosphate transport system, thus supporting the above conclusion.

The doubling time (100 min) shown in this figure is comparable with one observed before the exposure to a non-permissive temperature. The synthetic activity of protein was restored promptly as soon as the culture was returned to 30° C. An essentially similar pattern of cell division was also obtained

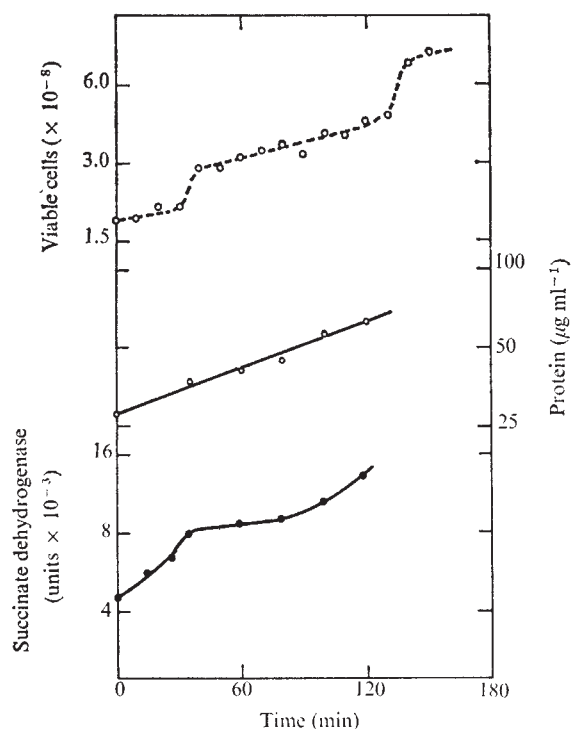


Fig. 5 Cell division and formation of succinate dehydrogenase after incubation at a non-permissive temperature in the mutant *tsC42*. Exponentially growing cells in A medium⁵ were shifted to 42°C and incubated for 35 min. Then the culture was returned to a permissive temperature (30°C) and the increases of viable cells (○---○) grown on a complete agar plate, succinate dehydrogenase (●) and total protein (○—○) were measured. Succinate dehydrogenase was measured according to the method described in the legend to Fig. 4 and units were expressed as μmol of DCIP reduced $\text{min}^{-1} \text{ml}^{-1}$. Total protein was measured according to the method in the legend to Fig. 1.

in the case of 90 min exposure to 42°C. These show that the exposure of cells to a non-permissive temperature had no vital influence and cells remained intact.

Our results indicate that either the conversion from inactive to active forms of the membrane components (or the co-ordinated expression of the genes responsible for cytochrome *b*₁ and so on), is under the control of a protein, which is only synthesised or activated 20–30 min before cell division.

Bacterial membranes possess many important biochemical functions such as energy production, active transport, lipid synthesis and cell wall synthesis. They are also special sites for chromosome segregation. To fulfil such complex and ordered roles, membranes are fabricated in a highly organised structural form. The simultaneous formation of many membrane components under the same regulatory mechanism may be an obligatory requirement for the fabrication of membrane units possessing such complex structures from individual precursor molecules. A characteristic feature of the mutant *tsC42* is that the mutation is pleiotropic and the control for cell growth is strict. This means that the functioning or the synthesis of a phase-specific protein is concerned with cell division. Double labelling experiments which discriminate old membrane components from newly-formed ones show that the incorporation of a radioactive amino acid into membrane components was specifically restricted to some membrane components only in non-permissive conditions (M.O., unpublished). Our preliminary experiment presents further evidence for our tentative conclusion that membrane proteins essential for cell growth and cell division are under the strict control of a phase-specific protein with a regulatory function for the formation of membrane components.

Recently Jones and Donachie⁹ have reported that cell division is controlled by a protein which is synthesised between termina-

tion of chromosome rounds and cell division. Both our and their results show that phase-specific proteins play important roles in cell division.

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Defect in the gamma polypeptide chain of a congenital abnormal fibrinogen (Paris I)

THE primary physiological function of fibrinogen is fibrin clot formation, a process that requires three distinct reactions. Thrombin first cleaves fibrinopeptides A and B from two of the three pairs of polypeptide chains ($\text{A}\alpha$, $\text{B}\beta$, γ) of fibrinogen¹, following which the remaining fibrin monomers polymerise spontaneously into an insoluble fibrin clot². Thrombin also converts Factor XIII to an active form, called plasma transglutaminase³, which in turn catalyses the formation of covalent γ -glutamyl- ϵ -lysine isopeptide bonds⁴ between two γ chains and more than two α chains of different fibrin molecules⁵. The resultant γ chain dimers and α chain polymers crosslink the fibrin network and render it resistant to chemical or physical modifications.

Any of these three reactions could be impaired by structural defects of fibrinogen, but most of the heritable disorders are associated with either a decreased rate of fibrinopeptide cleavage by thrombin or defective polymerisation of fibrin monomers^{6,7}. To date, a specific structural defect has been documented in only one abnormal fibrinogen, Fibrinogen Detroit⁸. The first well-studied abnormal fibrinogen, Fibrinogen Paris I⁹, showed a strikingly decreased ability to form fibrin polymers after reaction with thrombin and also inhibited the polymerisation rate of normal fibrin monomers. Studies of the fibrin monomers¹⁰ suggested the presence of two populations of fibrinogen molecules in the propositus. Chemical analyses showed that fibrinopeptides A and B⁶, the NH_2 -terminal amino acids⁸, and the $\text{A}\alpha$ chain of the NH_2 -terminal disulphide knot¹¹ of Fibrinogen Paris I are normal. Here we describe experiments which demonstrate that Fibrinogen Paris I possesses an abnormal γ chain, which is probably the cause of the impaired polymerisation of fibrin monomers. In addition, a second functional defect of this molecule will be documented, namely, its inability to crosslink in the presence of Factor XIII.

Fibrinogen (Fraction 1-0) was prepared by the technique of Blombäck and Blombäck¹² from normal citrated plasma and from citrated plasma of the propositus, in the presence of trypsin inhibitor (Iniprol, Choay, Paris). Factor XIII was obtained from fresh citrated bovine plasma according to the method of Loewy *et al.*¹³. Human thrombin was supplied by Dr David L. Aronson, Bureau of Biologics, Food and Drug Administration, Rockville, Maryland.

The α and β chains of non-crosslinked normal and Paris I

fibrin have the same electrophoretic mobility (Fig. 1, gels 1 and 2) and in both cases represent 30% and 36%, respectively, of the total protein, as calculated from densitometric scans (Densicord 532, Photovolt Co., New York). But, the band in the position of normal γ chain stains much lighter in Paris I fibrin, and represents only 12% of the total protein compared with 34% in the normal fibrin. In addition, Paris I fibrin has an extra band between the positions of the normal β and γ chains representing 22% of the total protein. From this, Paris I fibrinogen probably contains two molecular populations of γ chain, of which about 35% is normal and about 65% is abnormal. The molecular weight of the abnormal variant of the γ chain is approximately $50,500 \pm 500$, as determined from its migration in SDS-polyacrylamide gel, using standards of normal α , β and γ chains, which have molecular weights of 68,000, 56,000 and 48,000, respectively¹⁵.

The electrophoretic pattern of reduced normal crosslinked fibrin shows the expected absence of γ and α chains and the presence of γ chain dimers and α chain polymers (Fig. 1, gel 3). Fibrin formed from Fibrinogen Paris I crosslinks only partially. The normal γ chain is absent and the γ dimer band is present (Fig. 1, gel 4). The abnormal $\gamma_{\text{Paris I}}$ chain persists in the Factor XIII-treated fibrin, apparently not participating in the formation of γ -glutamyl- ϵ -lysine crosslink bonds. The concentration of α chain in Factor XIII-treated Paris I fibrin is less than in non-crosslinked fibrin. But $66\% \pm 15\%$ of the α chains still remain in monomeric form, compatible with the small amount of α chain polymers present in Factor XIII-

treated Paris I fibrin (Fig. 1, gel 4). The amount of α chain which does not participate in polymer formation correlates well with the proportion of γ chains that are abnormal ($\gamma_{\text{Paris I}}$). Since the α chains in Paris I fibrin are otherwise normal in appearance, this suggests that the failure of the abnormal $\gamma_{\text{Paris I}}$ chains to form dimers prevents the α chains of the same molecule from undergoing polymerisation. Thus, of the two varieties of fibrinogen in the plasma of the propositus, only those molecules with normal γ chains undergo cross-linking of both the γ and the α chains. This is supported by determination of the urea-soluble fibrin present in Factor XIII-treated Paris I fibrin. Overnight extraction with 5 M urea dissolves approximately 60% of the total fibrin, correlating well with the proportion of γ and α chains in Paris I fibrin that does not crosslink. These changes are not the result of a Factor XIII defect, since this activity was normal in both the propositus and his father⁶.

The abnormal $\gamma_{\text{Paris I}}$ chains are about 2,500 daltons heavier than the normal γ chains, corresponding to an additional polypeptide chain of about 22 amino acid residues. Since it is known that crosslinking of γ chains involves the glutamine and lysine residues in positions 14 and 6, respectively, from the carboxy-terminal end¹⁶, and since the NH_2 -terminal amino acids of Fibrinogen Paris I are the same as those of normal fibrinogen⁶, the molecular defect appears to involve the COOH-terminal region. The heavier molecular weight of the $\gamma_{\text{Paris I}}$ chain suggests that the structure is so altered as to impair the proper alignment of fibrin monomers for both polymerisation and Factor XIII-induced stabilisation. As such, this is the first documented example of an abnormal fibrinogen which fails to crosslink.

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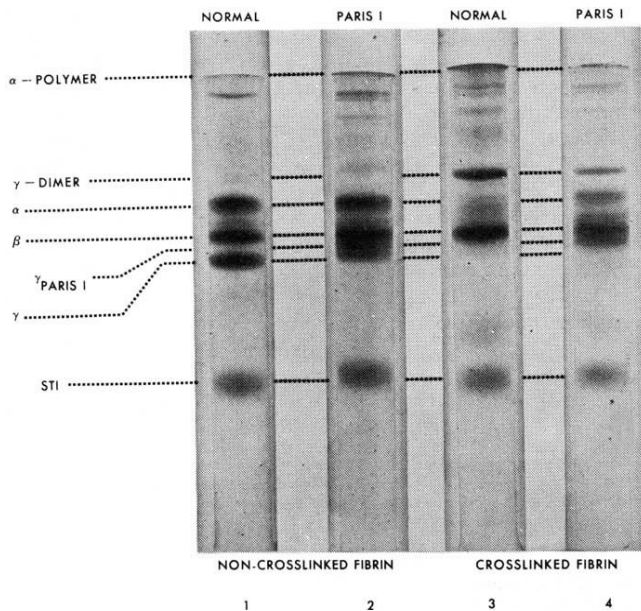


Fig. 1 The electrophoretic patterns in SDS-polyacrylamide gels of reduced fibrin samples obtained from purified normal human fibrinogen and Paris I fibrinogen. Non-crosslinked fibrin was obtained by adding 0.01 ml of human thrombin (100 NIH units ml^{-1}) to a mixture of 0.1 ml fibrinogen (4 mg ml^{-1}), 0.01 ml of soybean trypsin inhibitor (10 mg ml^{-1}) (Sigma), 0.01 ml of freshly purified bovine Factor XIII (2 mg ml^{-1}) and 0.01 ml of 0.1 M EDTA. Crosslinking of fibrin was accomplished by adding thrombin to a similar reaction mixture, except that 0.01 ml of 0.01 M calcium chloride was substituted for the EDTA. Reaction mixtures were incubated at 37°C for 24 h and then at 0°C for 1 h. After incubation, 0.015 ml of ethanol was added and the clots were dissolved by adding 0.5 ml of a solution containing 9 M urea, 3% sodium dodecyl sulphate and 3% β -mercaptoethanol and incubating at 37°C for 2 h. Aliquots of 5 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis, performed according to the method of Weber and Osborn¹⁴, and gels were stained with Coomassie Brilliant Blue^{14,15}. The rapidly migrating band present in the lower portion of each gel is soybean trypsin inhibitor (STI), which was added as an internal electrophoretic standard.

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Carcinogenic polynuclear hydrocarbons bind to macromolecules in cultured human bronchi

THE ability of cells in human tissues to metabolise procarcinogens into oncogenic metabolites may be one important determinant in organ and host susceptibility to environmental carcinogens. Metabolic activation of polynuclear aromatic hydrocarbons (PNH) seems to be required before they can cause neoplastic transformation of cells either *in vivo* or *in vitro*^{1,2}. This activation may involve the production of a metabolite(s) capable of tight binding to cellular macromolecules including DNA. The binding level of PNH to macromolecules generally shows a good correlation with their oncogenicity in experimental animals and cells in culture³⁻⁶, although exceptions exist⁷. Whether or not this correlation exists in man is unknown.

We report here that human bronchial epithelial cells in organ culture have the capability to bind and presumably to activate PNH [7.12-dimethyl benz(a)anthracene (DMBA); 3-methylcholanthrene (MCA); benzo(a)pyrene (BP) and dibenz(a,h)anthracene (DBA)]. Binding to DNA was measured by biochemical methods while total binding to epithelial cell macromolecules was shown by autoradiography. These methodologies had been previously developed in studies utilising an animal model of human lung cancer^{8,9}.

Bronchi were obtained as surgical specimens from three patients with lung cancer and from one patient without lung cancer. Grossly normal-appearing pieces of bronchi were immediately immersed in L15 medium¹⁰ at 4°C until cultured. The bronchi were then cut into approximately 1×1 cm squares and placed in 60 mm plastic Petri dishes with the epithelium oriented toward the gas-liquid interface. Two ml of CMRL 1066 medium¹¹ containing 1 µg ml⁻¹ crystalline insulin, 0.1 µg ml⁻¹ hydrocortisone hemisuccinate, 0.1 µg ml⁻¹ β-retinyl acetate, 100 units ml⁻¹ penicillin G, 2 mM L-glutamine and 100 µg ml⁻¹ streptomycin were added to each dish¹⁷. Cultures were maintained in an air-tight box and gassed with 50% O₂, 45% N₂ and 5% CO₂ for 5 min. The medium was replaced and the cultures were gassed every 2 d. The box containing the cultures was rocked in the dark at approximately 10 cycles min⁻¹ so that the bronchial tissue was submerged half of the time. Cultures were incubated at 36.5°C.

At 7 d, tritium labelled PNH (³H-DMBA, 14.5 Ci mmol⁻¹, (Amersham/Searle); ³H-MCA, 5 Ci mmol⁻¹ (Amersham/Searle); ³H-BP, 17 Ci mmol⁻¹, (Amersham/Searle) and ³H-DBA, 2.42 Ci mmol⁻¹ (New England Nuclear)) were dissolved in dimethyl sulphoxide (final concentration 0.5%) and added to the culture medium at a final concentration of 40 µCi ml⁻¹. The radiopurity of these compounds was determined by radioscan of thin layer chromatograms. When necessary the compounds were re-purified by chromatography⁸ to a radiopurity of greater than 99%. Following incubation for 24 h, the bronchial tissues were washed three times with cold Dulbecco's phosphate-buffered saline. For autoradiography four cultures per experimental variable were fixed overnight in 3% glutaraldehyde buffered by 0.1 M *s*-collidine, pH 7.4, and then postfixed in 1.33% OsO₄ in the same buffer. As

Table 1 Specific activities of binding of tritium-labelled polynuclear hydrocarbons to human bronchial DNA

Polynuclear hydrocarbon	Specific activity* (d.p.m. per µg DNA)	(pmol per mg DNA)
DMBA	170±22† (3)‡	53±7
BP	224±77 (4)	40±14
MCA	38±9 (2)	34±8
DBA	15±3 (3)	28±6

* The amount of DNA and d.p.m. were determined from the peak DNA fraction of CsCl gradients. See text for specific activities (Ci mmol⁻¹) of tritiated PNH.

†Mean ± s. e.

‡Number of cases studied.

previously described⁹, tissues were repeatedly washed in dehydrating solutions of ethanol to remove unbound PNH; The radioactivity released into the dehydrating solutions was monitored by liquid scintillation methods. The tissues were then embedded in Epon 812 containing 5% Araldite 502, 1 µm sections were cut, and autoradiograms were prepared¹².

Radioactivity was found in both the nuclei and cytoplasm of epithelial cells incubated with ³H-DMBA (Fig. 1). Ciliated, mucous and basal cells contained tightly bound radioactivity. Epithelial cells in bronchial glands were overlaid also with autoradiographic grains. Similar results were found with ³H-BP, ³H-MCA and ³H-DBA. Quantitative analysis of the autoradiograms will be reported fully elsewhere. Bound radioactivity was also found in mesenchymal cells, but to a lesser extent. This observation is consistent with the finding that human embryonic lung fibroblasts are capable of activation and binding of PNH *in vitro*¹³. For biochemical studies, bronchial mucosa cells were scraped from the supporting cartilage. Cells from six cultures were pooled for each carcinogen-case combination. DNA was isolated from these cells, and the results of CsCl gradients of the purified DNA are shown in Fig. 2 and Table 1. In the cases studied to date, DMBA and BP bind to DNA more than DBA.

We wished to determine if a major target tissue of environmental carcinogens, the human bronchus, had the capability to activate PNH into forms which tightly bind to macromolecules. It is apparent that the human bronchial mucosa has this capability after it has been maintained

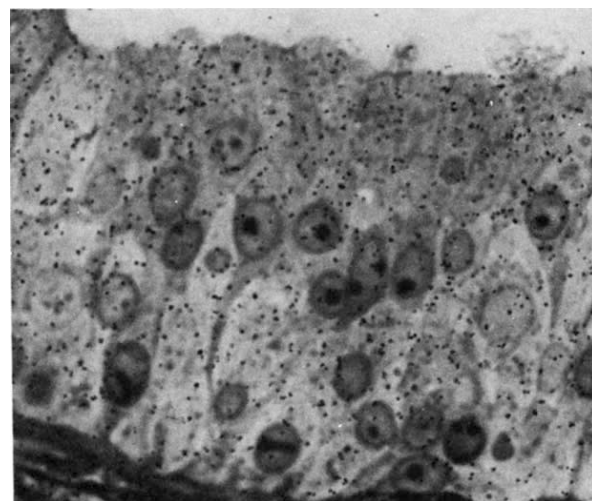


Fig. 1 Autoradiogram of human bronchial epithelial cells following *in vitro* incubation with ³H-DMBA (40 µCi ml⁻¹) for 24 h. Autoradiographic grains are found overlying both nuclear and cytoplasmic sites. One micrometre section stained with toluidine blue (×880).

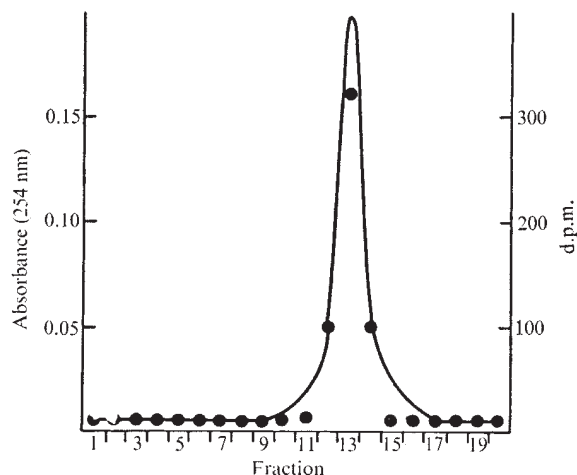


Fig. 2 CsCl gradient profile of DNA purified from human bronchial mucosa. After 7 d in organ culture, bronchial pieces were incubated with ^3H -MCA ($40 \mu\text{Ci ml}^{-1}$) for 24 h. The bronchial mucosa containing epithelial and mesenchymal cells was then scraped from supporting structures and a crude preparation of DNA was obtained by phenol extraction and precipitation with 50% ethanol. The precipitate was repeatedly extracted with ether and benzene to remove unbound tritiated PNH and sequentially digested with RNase (Worthington Biochemicals) and Pronase (Calbiochem). The aqueous solution of DNA was extensively dialysed and then banded on CsCl gradients (Beckman SW56 rotor, 35,000 r.p.m.; 66 h, 25°C). Gradients were fractionated into 0.2 ml portions, and absorbance and radioactivity were determined on each fraction. The absorbance (—) was continuously monitored at 254 nm; radioactivity (d.p.m.) is indicated by the points. Each gradient contained approximately $10 \mu\text{g}$ of DNA.

in organ culture for 7 d. This finding is consistent with previous studies^{14,15} showing morphological changes in human respiratory epithelium caused by PNH *in vitro*.

As this system becomes better defined, it may be used to determine the individual variations of binding activity in groups at different risks to bronchogenic carcinoma; in addition this system may be useful in evaluating experimental attempts to modify and inhibit the activation of PNH in the human bronchial epithelium. Studies comparing the level of PNH binding to DNA in a large number of individuals with and without lung cancer are needed. If groups at high risk to develop lung cancer can be identified then steps to intervene can be taken¹⁶. We thank William Kaufmann, Frank Jackson and Maria Yamaguchi for technical assistance. The comments of Drs Yuspa, Sporn, Saffiotti and Janss were appreciated by the authors. This work was supported in part by a National Cancer Institute contract.

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Dose-related inhibition of chemical carcinogenesis in mouse skin by caffeine

THERE is some evidence that caffeine protects against the effects of chemical carcinogens^{1,2}. Leitner and Shear³ found a reduction in the effectiveness of benzo(a)pyrene as a carcinogen when it was administered by subcutaneous injection together with various purines, including caffeine. An enhancement of the antitumour effect of 1,3-bis(2-chloroethyl)-1-nitrosourea by caffeine has been reported⁴ but whether the caffeine acts as an anticarcinogen or as a membrane-active agent (a property of caffeine which has been demonstrated⁵) is not clear. Tumour production *in vivo* has been shown to be partially suppressed by theophylline⁶ and it is possible that caffeine which is chemically closely related to theophylline may similarly suppress tumour production.

We have shown that caffeine inhibits the carcinogenic action of cigarette smoke condensate fractions when added to the fractions before application. Our experiments were prompted by unexpected results obtained when testing fractions of cigarette smoke condensate for carcinogenicity. Traces of caffeine, remaining after a fractionation procedure involving caffeine⁷, were found in fractions which showed reduced carcinogenicity when applied to mouse skin. The effect of caffeine was then tested systematically.

Two fractions of smoke condensate, from plain cigarettes manufactured from flue-cured tobacco representing the major plain cigarette brands smoked in the United Kingdom, were prepared by standard methods^{7–9} and applied repeatedly to mouse skin at two dosages either alone or together with caffeine, also at two concentrations. Dose levels of condensate fractions refer to the amount of smoke condensate from which the fraction was derived; thus an application of 150 mg of a fraction which represented 25% by weight of the parent condensate is referred to as a 600 mg dose. All treatments were given as solutions/suspensions in 0.3 ml of isopropanol: acetone (20:80) three times weekly until the last mouse died (112 weeks). Procedures used for animal husbandry, skin shaving, the application of fractions, post mortem and histopathological examination of mouse skin were as previously described^{8,10}.

The two fractions tested were: first, fraction G, a cyclohexane soluble fraction, prepared from smoke condensate by removal of water soluble materials followed by distribution of the water insoluble phase between 90% methanol and cyclohexane⁸. This fraction, representing 25% by weight of the original condensate, accounted for 80% of the carcinogenic activity of the condensate. Second, fraction RP prepared by extracting a cyclohexane solution of fraction G with a 15% w/v solution of caffeine in 90% formic acid followed by removal of the caffeine⁷. This fraction, representing 3.3% by weight of

Table 1 Effect of topically applied caffeine on the incidence of skin tumours in mice in response to fractions of cigarette smoke condensate applied to the skin

Condensate fraction	Treatment applied thrice weekly in 0.3 ml solvent for life			No. of animals per group	Tumour-bearing animals		Infiltrating-carcinoma bearing animals	
	Actual dose per application (mg)	Equivalent dose of condensate (mg)	Dose of caffeine per application (mg)		Relative incidence rate $b \times 10^6$ ($k=3, w=15.26$)	%	Relative incidence rate $b \times 10^{12}$ ($k=6, w=12.55$)	%
G	25	100	0	51	37	2.4	14	1.3
G	50	200	0	51	61	8.5	35	4.3
G	25	100	0.04	51	57	3.9	14	1.4
G	50	200	0.08	51	67	6.7	20	2.6
G	25	100	0.20	51	41	2.3	12	0.9
G	50	200	0.40	51	47	3.6	20	2.3
RP	3.3	100	0	51	51	3.7	18	1.8
RP	6.6	200	0	51	75	7.3	31	4.1
RP	3.3	100	0.04	51	24	1.6	8	1.0
RP	6.6	200	0.08	51	61	5.8	28	5.0
RP	3.3	100	0.20	51	37	2.4	10	1.0
RP	6.6	200	0.40	51	47	3.6	16	1.7
Caffeine	0	—	0.08	51	0	0.0	0	0.0
Caffeine	0	—	0.40	51	2	0.1	0	0.0
Untreated control	0	—	0	102	2	0.1	0	0.0
Solvent control	0	—	0	102	2	0.1	0	0.0

the original smoke condensate, accounted for more or less all the carcinogenic activity of the parent fraction, G. The concentration of caffeine added to the mouse painting solutions were: 0.04% of the smoke condensate equivalent (that is, 0.24 mg week⁻¹ for a 600 mg dose and 0.12 mg week⁻¹ for a 300 mg dose) or 0.2% of the smoke condensate equivalent (that is 1.2 mg week⁻¹ for a 600 mg dose and 0.6 mg week⁻¹ for a 300 mg dose). Caffeine was also tested alone at doses of 0.24 mg week⁻¹ and 1.2 mg week⁻¹.

The results, given in Table 1, are presented as percentage of animals bearing one or more benign or malignant tumours (TBA), percentage of animals bearing carcinomas showing invasion of the panniculus muscle on microscopic examination (CBA) and the relative incidence rates for tumours and carcinomas.

The relative incidence rate parameter, b , is obtained by the fit of a Weibull distribution which postulates that the incidence rate at time t , in treatment group i , is given by the expression $b_i k(t-w)^{k-1}$ in which k and w are independent of the treatment¹¹. The relative incidence rate is a better measure of effect than are simple percentages as it compensates for different mortality rates in the groups.

From the results it is clear that caffeine alone did not lead to the development of skin tumours. To evaluate the effect of the three variables (fraction, dose and caffeine) in the other groups, the method of Weibull multiple regression was used¹². The analysis showed that there was a highly significantly greater response ($P < 0.001$ for both TBA and CBA) at 600 mg dose levels than at 300 mg dose levels. In fact the response at 600 mg was, on average, 2.2 times greater than at 300 mg for tumours and was 2.6 times greater for carcinomas. At the higher caffeine concentration, the caffeine significantly reduced tumour incidence ($P < 0.001$ for TBA and < 0.005 for CBA). At the lower caffeine concentration, there was also a reduction in tumour incidence but the effect was not statistically significant. There was no evidence of a difference in response between the two fractions, nor of any interaction between the three factors: fraction, dose of fraction and concentration of caffeine.

Although it is not known how caffeine brings about these effects, it is known that purines form complexes with polycyclic aromatic hydrocarbons (PAH) and heteropolycyclic compounds. Both these chemical classes have representative compounds in cigarette smoke condensate and some members of both classes are chemical carcinogens. The complexing is relatively weak in the case of the PAH; resulting from a dipole-induced dipole interaction between the polar component (caffeine) and the polarisable component (the aromatic compound).

Attempts have been made to relate complexing ability of PAH to their carcinogenic properties, but none has been successful^{1,2}. It is nevertheless possible that the complexing property of PAH makes an important contribution to their carcinogenicity, the differences in their activity resulting from a combination of factors such as molecular size, geometry and general reactivity. Thus, the absence of carcinogenic activity in the majority of PAH might arise from diffusion and transport difficulties within the cell or a lack of ability to adapt to a favourable configuration at the site of action within the cell. The inhibition of carcinogenesis by caffeine could result from competition for the polycyclic compound between the site of action in the cell and the caffeine.

The possibility that caffeine may inhibit transport of PAH through cell membranes cannot be overlooked, but caffeine can facilitate the transport of some compounds through membranes⁵, so this explanation seems unlikely.

Both caffeine and theophylline are known to affect the level of adenosine 3', 5'-cyclic monophosphate (cyclic AMP) in mammalian cells¹³. It has been suggested that a partial suppression of tumour production by theophylline was due to an effect on cyclic AMP synthesis⁶ and caffeine may inhibit tumour production by a similar mechanism. If this were the correct explanation, caffeine could be expected to act as an inhibitor of chemical carcinogenesis in general and not specifically of polycyclic hydrocarbon induced carcinogenesis. This possibility can therefore be tested.

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Caffeine inhibits induction of endogenous C-type virus

ENDOGENOUS C-type viruses can be induced by treating the rodent cells in which they reside with halogenated pyrimiding analogues, notably 5-iodo-2'-deoxyuridine (IUdR) (refs 1, 2). Here I show that caffeine, which inhibits repair of photolesions and interacts with DNA in various ways³, inhibits the induction of the endogenous virus by IUdR.

The MLg cell line originating from a newborn ddY mouse was used; this cell line was found to release C-type virus after IUdR treatment⁴. The induced virus was detected by the XC test⁵.

The frequency of virus production was estimated as follows. MLg cells inoculated in 60 mm plastic Petri dishes (10^5 cells per dish) were treated with IUdR ($5\text{--}50\text{ }\mu\text{g ml}^{-1}$) for 24 h; the medium was removed and untreated MLg cells (10^5 cells per dish) were laid over the IUdR-treated cells. Three days later, three subcultures were made; after a further 3 d, the cultures were submitted to the XC plaque titration test. For IUdR concentrations of 5, 20 and $50\text{ }\mu\text{g ml}^{-1}$ the mean plaque number per dish was 19, 73 and 125, respectively. A proportional relation between plaque number and IUdR concentration was observed. There was some variation of induction frequency from one experiment to another, but the variation was small within one experiment, (see Fig. 1a). In the subsequent experiments, the concentration of IUdR was used at $25\text{ }\mu\text{g ml}^{-1}$ for 24 h.

The cells were treated with caffeine (0.625 to 5 mM) for 24 h before or after IUdR treatment ($25\text{ }\mu\text{g ml}^{-1}$ for 24 h). Twenty-four hours after the removal of IUdR, the cultures were overlaid with normal MLg cells (10^5 cells per dish). Three days later, each culture was divided into three, and

submitted to the XC test. Figure 1a shows that caffeine treatment after IUdR treatment significantly inhibited virus induction; a proportional relation was observed between caffeine concentration and the inhibition of induction of the C-type virus. Treatment with doses of caffeine as high as 2.5 mM before IUdR treatment was almost without effect.

In order to test the effect of caffeine on the exogenous C-type virus infection, MLg cells were treated with caffeine (1.25 to 5 mM) for 24 h before or after infection with Friend murine leukaemia virus (adsorption for 2 h). Twenty-four hours after infection, 10^5 normal MLg cells were added per dish. Three days later, the XC test was performed. Figure 1b shows that caffeine was without effect on exogenous virus infection.

Various ways of inhibiting cellular DNA synthesis, such as treatment with arabinosyl cytosine, excess thymidine and serum starvation, have been reported to inhibit virus induction⁶. The inhibition was presumably due to inhibition of IUdR incorporation. These conditions also inhibit exogenous C-type infection^{7,8}; consequently, in some cases, propagation of the induced virus may be inhibited.

Since caffeine affects neither the exogenous virus infection nor incorporation of ^{125}I IUdR (data not shown) caffeine may interfere with events specific to virus induction.

What happens between IUdR incorporation into the cellular DNA and the subsequent release of the induced virus is obscure. Excision of the viral genome may or may not be necessary. In any event, viral induction may be brought about by some toxic modifications of DNA by IUdR. It is possible that IUdR may induce local distortion of DNA as the halogenated pyrimidines when incorporated into DNA cause fragmentation or instability of DNA⁹. Caffeine, known to interact with denatured but not with helical DNA⁹, may interact with such distorted regions of the DNA, so as to prevent viral gene activation. This last suggestion gains support from the fact that caffeine was especially effective after IUdR incorporation.

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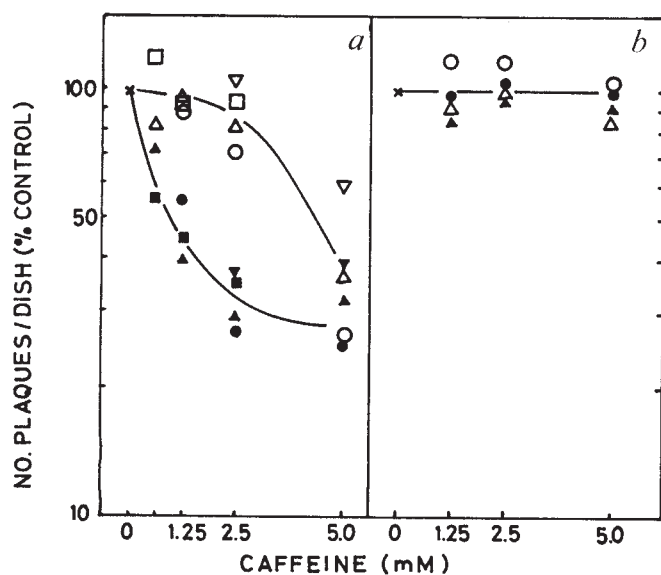


Fig. 1 a, Effect of caffeine on endogenous C-type virus induction. Closed symbols indicate caffeine treatment after IUdR ($25\text{ }\mu\text{g ml}^{-1}$), and open symbols indicate caffeine treatment before IUdR. Four repeated experiments are shown. The mean number of plaques per dish was obtained from three cultures which were derived from one plate. In some experiments, 2 cultures were prepared for the control; the mean plaque number in this case is the mean of the two values obtained. The mean plaque number of the control was 129 for experiment 1 ($\circ$, $\bullet$), 134 and 119 for experiment 2 ($\triangle$, $\blacktriangle$), 114 and 98 for experiment 3 ($\square$, $\blacksquare$), and 95 and 85 for experiment 4 (∇ , $\blacktriangledown$). **b**, Effect of caffeine on exogenous Friend leukaemia virus infection. Open symbols indicate treatment before infection and closed symbols after infection. Duplicate experiments are shown. Three dishes were used for each point. The mean plaque number in the control dishes was 125 and 109 for experiment 1 ($\circ$, $\bullet$) and experiment 2 ($\triangle$, $\blacktriangle$), respectively.

Isolation of an inhibitor protein of *E. coli* RNA polymerase from T7 phage infected cell

BACTERIOPHAGE T7 gene expression is under a unique transcriptional control. The 'early to late' switch in T7 development involves a switch from the host *Escherichia coli* RNA polymerase to the T7-coded enzyme (T7-specific RNA polymerase¹), transcribing early mRNA and late mRNA, respectively, from two different regions of the T7 genome². In addition to the gene 1 coding for T7-specific

RNA polymerase, this early-late switch requires the function of another early gene of T7, gene 0.7 or the 'host shut-off' gene^{2,3}, to shut off the synthesis of host RNA and T7 early mRNA. This shut-off function is assumed to be an inactivation of the host RNA polymerase, although this has not been proven^{4,5}. Furthermore, we have previously shown that T7 early mRNAs are functionally unstable, although they remain chemically stable late in T7 infection^{6,7}. Thus, it seems that a complete switch of T7 gene expression from early to late involves at least three different control mechanisms. It has been shown that a T7-coded translational repressor may also play a role in the elimination of host functions⁸.

Here we report that the 'host shut-off' function is indeed an inactivation of the host RNA polymerase molecule, involving an inhibitor protein bound to the enzyme. When this inhibitor protein, here referred to as 'I' protein, is removed from the inactive host RNA polymerase prepared from T7 infected cells, the core enzyme and the sigma factor are both functionally active. The I protein, with a molecular weight of about 66,000, has been purified. It inhibits the host RNA polymerase holoenzyme, but does not inhibit the core enzyme or the T7-specific RNA polymerase.

Figure 1 shows the switch from the host RNA polymerase to the T7-specific enzyme in normal T7 infection. Cell-free extracts were prepared from T7 infected cells at the times indicated and assayed for the two enzymes. A rapid reduction of the host RNA polymerase activity coincided with a rapid increase of the T7-specific RNA polymerase activity. Since addition of rifampicin 5 min or later after T7 infection did not change the kinetics of appearance or the final yield of T7-specific RNA poly-

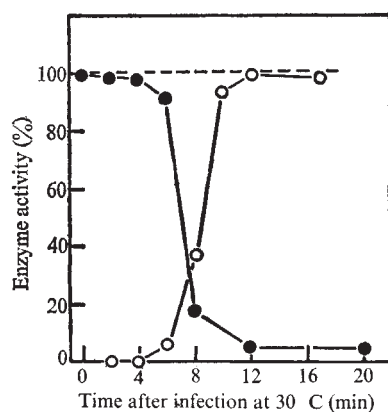


Fig. 1 Inactivation of *E. coli* RNA polymerase and appearance of T7-specific RNA polymerase after T7 infection. *E. coli* D10 F⁻ (ref. 6) was infected with T7 phage at a multiplicity of infection of 5 at 30°C. Cells were also infected with T7 in the presence of chloramphenicol (150 µg ml⁻¹) added 5 min before infection. Aliquots of the culture were withdrawn at indicated times and the cells were collected by centrifugation. The cells were suspended in a buffer containing 0.05 M Tris-HCl, pH 7.9, 0.01 M MgCl₂, 0.15 M KCl, 1 mM dithiothreitol (DTT) and 0.1 mM EDTA and sonicated. Sonicated extracts were centrifuged at 12,000g for 10 min. Approximately 400 µg (protein) of the cell-free extract was used for the assay of *E. coli* RNA polymerase activity. The standard assay mixture¹⁹ contained 5 µg T4 DNA in a total volume of 0.25 ml and was incubated at 37°C for 15 min. The sonicated cell-free extract was also used for the assay of T7-specific RNA polymerase activity following the method previously described^{4,7}. The assay mixture contained 5.6 µg T7 DNA and 5 µg rifampicin in a total volume of 0.25 ml, and was incubated at 37°C for 10 min. Since rifampicin inhibits only *E. coli* RNA polymerase, this assay is specific for T7-specific RNA polymerase. Enzyme activities were normalised by measuring the protein content of each extract. The 100% values were: 5,100 c.p.m. ³H-UTP incorporated per mg protein for *E. coli* RNA polymerase, and 62,000 c.p.m. ³H-UTP incorporated per mg protein for T7-specific RNA polymerase. (●), *E. coli* RNA polymerase; (---), *E. coli* RNA polymerase in the presence of chloramphenicol; (○), T7-specific RNA polymerase.

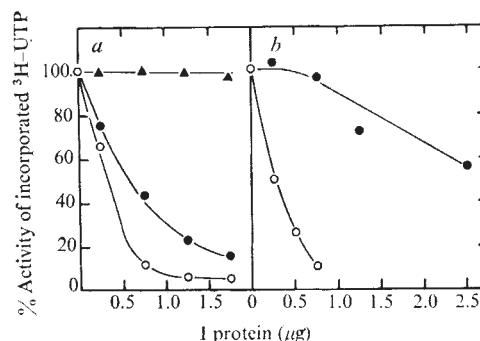


Fig. 2 Specific inhibition of *E. coli* RNA polymerase holoenzyme by I protein. *a*, Standard RNA-synthesising system¹⁹ contained ³H-UTP and 2.4 µg T4 DNA or 4.4 µg poly(dAT), and was incubated at 37°C for 15 min. Various amounts of purified I protein, electrophoretically more than 90% pure, were added to the reaction. (○), *E. coli* RNA polymerase holoenzyme (4.4 µg) on T4 DNA. The 100% value was 23,000 c.p.m. per reaction. (●), *E. coli* RNA polymerase holoenzyme (4.4 µg) on poly(dAT). The 100% value was 35,000 c.p.m. per reaction. (▲), *E. coli* RNA polymerase core enzyme (3 µg) on poly(dAT). The 100% value was 45,000 c.p.m. per reaction. *b*, *E. coli* RNA polymerase holoenzyme was assayed as in *a* with 4.5 µg T7 DNA. T7-specific RNA polymerase was assayed as in Fig. 1, with 4.5 µg T7 DNA in the presence of rifampicin^{1,7}. The actual molar amount of T7-specific RNA polymerase protein used was about one-twentieth of that of *E. coli* RNA polymerase. Increasing amounts of I protein were added. (○), *E. coli* RNA polymerase on T7 DNA. The 100% value was 26,000 c.p.m. per reaction. (●), T7-specific RNA polymerase on T7 DNA. The 100% value was 27,000 c.p.m. per reaction.

merase⁷, we assume that no new initiations of T7 early mRNA transcription by the host RNA polymerase occur after this time (T7-specific RNA polymerase is one of the early gene products²). This shut-off of T7 early mRNA synthesis, probably concomitant with the shut-off of host RNA synthesis, parallels with the decline of the host RNA polymerase activity (Fig. 1). When the cells were infected with T7 in the presence of chloramphenicol, no reduction in the activity of the host RNA polymerase was observed, indicating that protein synthesis after T7 infection is required to accomplish the shut-off function.

The result shown in Table 1 was obtained after removing endogenous DNA from sonicated cell-free extracts by DNase treatment. DNase-treated extracts were centrifuged in glycerol gradients and the lower portions of the gradients, containing the host RNA polymerase activity but free of DNase activity, were pooled and assayed for RNA synthesis on T4 phage DNA and poly(dAT) templates. The crude enzyme from uninfected cells exhibited an activity of above 1.2 on T4 DNA relative to that on poly(dAT) and this activity ratio, T4 DNA: poly(dAT), reached about 2.0 after purification. The crude host enzyme from T7 infected cells however, showed an activity ratio of only about 0.2. A considerable activity on poly(dAT) in the enzyme from T7 infected cells was used to monitor its recovery during purification. The presence of chloramphenicol prevented the reduction of the host RNA polymerase activity in T7 infected cells (Table 1).

The *E. coli* RNA polymerase was purified from uninfected control cells and T7 infected cells harvested 9 min after infection, using essentially the same procedure described by Burgess⁹. After the first glycerol gradient purification step in the absence of KCl, the control enzyme showed an activity ratio on T4 DNA against poly(dAT) of between 1.5 and 2.0, whereas the enzyme from T7 infected cells showed a ratio of about 0.2. After the second glycerol gradient step in the presence of 1 M KCl, the relative activity on T4 DNA and poly(dAT) of the control enzyme was about 2.0, while the enzyme from T7 infected cells displayed a value about 1.0. The increased activity of the enzyme from T7 infected cells on T4 DNA after

the second gradient step was due to a partial removal of inhibitory material. When gradient fractions sedimenting more slowly than the enzyme were added back to it, its relative activity on T4 DNA and poly(dAT) was about 0.2, the same as that before the second gradient step.

The purified enzymes, after the second gradient step, were analysed by electrophoresis in polyacrylamide-SDS slab gels. The enzyme from T7 infected cells contained, in addition to α , β , β' subunits and sigma factor, two protein components which were usually absent in the control enzyme and when present, only in a trace amount. One protein component tentatively termed as I has been purified as described below, and found to be an inhibitor of the host RNA polymerase. The other protein migrating slightly faster than the I protein has also been purified, but has no effect on the RNA polymerase activity.

The RNA polymerase from T7 infected cells recovered full sigma and core enzyme activities when the inhibitor protein I was completely removed. This was shown by subjecting the host RNA polymerase from T7 infected cells to phosphocellulose column chromatography. The protein I was separated from the enzyme complex and eluted in the flow-through. The sigma factor and core enzyme were also separated. Sigma factor was further purified by DEAE column chromatography and glycerol gradient centrifugation. The purified sigma factor and the core enzyme eluted from the phosphocellulose were electrophoretically more than 95% pure. Reconstitution experiments showed that purified sigma factor and core enzyme from T7 infected cells are functionally normal in forming fully active RNA polymerase holoenzyme both with each other, and with samples purified from uninfected cells.

The I protein was purified from the flow-through of the phosphocellulose column and by DEAE cellulose column chromatography followed by glycerol gradient centrifugation. Purified I protein moved slightly faster than bovine serum albumin in electrophoresis in the presence of SDS and its molecular weight was estimated as about 66,000. In some cases it was purified from the enzyme preparation after the first glycerol gradient, because at this step the enzyme contained more I protein than after the second gradient.

Figure 2a shows the inhibition of *E. coli* RNA polymerase holoenzyme by purified I protein. RNA synthesis on T4 DNA was inhibited almost completely at a molar ratio of I to enzyme of about 1. The holoenzyme activity on poly(dAT) was also inhibited by I, but to a slightly lesser extent. No inhibition was observed when I protein was added to the core enzyme synthesising RNA on poly(dAT). Figure 2b also shows the inhibition of the holoenzyme by purified I protein in T7 DNA-directed RNA synthesis. At a concentration of I protein (0.75 μ g) which inhibited the host RNA polymerase by 90%, T7-specific RNA polymerase was not inhibited. At higher I protein concentrations, T7-specific RNA polymerase was somewhat inhibited. Because the molar amount of T7-specific RNA polymerase used in the reaction was about one-twentieth of that of the host enzyme, however, at these concentrations of I protein the ratio of I to the T7-specific RNA polymerase was very high.

These results indicate that the inhibition by I protein is specific for the host RNA polymerase holoenzyme and that the presence of sigma factor is required for this inhibition. The details of the interaction between I protein and the holoenzyme are under investigation. Since the core RNA polymerase of the host and the T7-specific RNA polymerase both synthesised normal amounts of RNA in the presence of I protein, at a concentration which effectively inhibited RNA synthesis by the host holoenzyme on the same templates (Fig. 2), I protein does not seem to have DNase or RNase activity. Also, T7 DNA-directed RNA synthesis by the host holoenzyme was inhibited by I protein

to a similar extent as shown in Fig. 2b even when four times more T7 DNA was added as template. This result suggests that DNase activity in I protein is unlikely and supports the idea that I protein interacts with the holoenzyme rather than with the DNA template.

We have not yet determined whether I protein is directly coded by T7, or is instead a host protein somehow activated or induced by T7 infection. It is certain, however, that protein synthesis after T7 infection is required to manifest the inactivation of the host RNA polymerase by I protein, since the presence of chloramphenicol permits the host RNA polymerase to remain fully active (Fig. 1, Table 1). If I protein is coded by T7, the most likely

Table 1 *E. coli* RNA polymerase activity after T7 infection

Conditions of T7 infection	Activity on T4 DNA (%)	Activity on poly(dAT) (%)	Ratio* T4 DNA / poly(dAT)
Uninfected	100†	100†	1.27†
9 min after infection	17	43	0.21
9 min after infection, (With chloramphenicol)	98	—	—

Sonicated cell-free extracts, prepared as in Fig. 1, were treated with 150 μ g ml⁻¹ DNase at 0° C for 15 min and centrifuged at 20,000g for 15 min. The extract was layered on a 15–30% glycerol gradient in 12 ml containing 0.01 M Tris-HCl, pH 7.9, 0.01 M MgCl₂, 0.15 M KCl, 1 mM DTT and 0.1 mM EDTA, and centrifuged at 200,000g for 12 h. Aliquots (40 μ l) of pooled bottom two-third fractions were assayed as in Fig. 1, with the addition of 3.4 μ g T4 DNA or 4.0 μ g of poly(dAT).

*In the activity ratio calculation, it was assumed that UMP appears twice as frequently in poly(dAT)-directed RNA as in T4 DNA-directed RNA. Therefore, the ratio is (pmol UMP in T4 RNA/mg protein $\times$ 2) : (pmol UMP in poly AU/mg protein).

†100% values were: 8,700 c.p.m. for T4 DNA, and 13,700 c.p.m. for poly(dAT).

‡Highly purified enzyme from uninfected cells usually showed a ratio about 2.0.

candidate is the 'host shut-off' gene (gene 0.7) as has been suggested³⁻⁵. Gene 0.7 transcribes mRNA of molecular weight 650,000 (refs 3 and 10), large enough to code for a protein of the size of I (molecular weight 66,000). The protein product of gene 0.7 is however known to have a molecular weight of only 40,000 although a precursor molecule of larger size may exist³. It is interesting that the product of gene 0.7 was recently shown to be a protein kinase which phosphorylates many host proteins¹¹. The possibility that I is a host protein cannot be ruled out. Indeed a protein similar to I in electrophoretic mobility was detectable, although in small amounts, in the RNA polymerase preparations from uninfected cells. It is also possible that gene 0.7 exerts 'host shut-off' function through the protein phosphorylating activity thereby directly or indirectly mobilising I protein.

With the closely related phage T3, a protein which inhibits the host RNA polymerase has been reported^{12,13}. Although this T3 protein seems to be controlled by a late gene, we cannot make a good comparison with I protein because the T3 protein has not been purified.

We have found in addition that I protein also inhibits *in vitro* translation of MS2 RNA. Figure 3 shows that I protein inhibited MS2 RNA-directed met-tRNA binding to ribosomes (Fig. 3a) and phenylalanine incorporation into protein (Fig. 3b). Using 1.5 μ g I protein and 5 μ g MS2 RNA, about 50% inhibition was achieved in both cases. Increasing amounts of MS2 RNA reduced the inhibitory effect of I protein; 10 μ g of MS2 RNA required about 3 μ g of I protein to be inhibited to the same extent. On the other hand, I protein did not inhibit *in vitro* translation (measured as above) of T7 early mRNA prepared from ultraviolet-irradiated, T7 gene 1-amber mutant phage-infected cells following the procedure described previously⁶. These characteristics are very similar to those of a cistron-

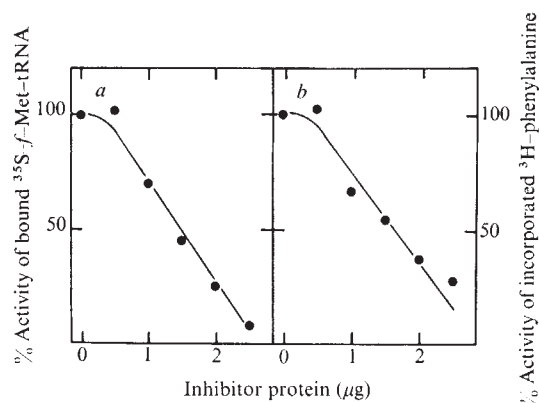


Fig. 3 Inhibition of *in vitro* translation of MS2 RNA by I protein. *a*, MS2 RNA-directed *f*-met-tRNA binding to ribosomes. The initiation factor-dependent reaction was carried out as described previously⁶ and contained 5 μg MS2 RNA, 75 μg NH₄Cl-washed ribosomes, 20 μg crude initiation factors and 3.5×10^5 c.p.m. ³⁵S-labelled *f*-met-tRNA. Various amounts of purified I protein were added as indicated. The reaction was at 35°C for 15 min. The 100% value was 52,159 c.p.m. per reaction. *b*, MS2 RNA-directed phenylalanine incorporation into protein. A standard *in vitro* protein-synthesising system⁶. It was confirmed that the major products of this reaction were MS2 coat protein and RNA replicase, by gel electrophoresis²⁰. The reaction was at 37°C for 15 min and contained 5 μg MS2 RNA, 10 μCi per 2 nmol ³H-phenylalanine and various amounts of purified I protein. The 100% value was 37,517 c.p.m. per reaction.

specific translational control protein, 'i' factor, which is also one of the host-coded subunits of Q_β phage RNA replicase (refs 14–18) and is probably identical with the *E. coli* 30S ribosomal protein, S1 (cited in ref. 16). The 'i' factor binds to the RNA of RNA phages MS2 (refs 14 and 15) and R17 (refs 16–18), and specifically inhibits translational initiation of the coat protein cistron, but it does not inhibit *in vitro* translation of T4 mRNA or endogenous *E. coli* mRNA. But 'i' factor seems to have a slightly larger molecular weight (74,000) than the I protein described here. Further investigation is in progress concerning the translational inhibition by I protein and its identity.

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Biological decay of the 5'-triphosphate termini of the RNA of *E. coli*

EXPERIMENTS *in vitro* and *in vivo* have shown that nascent RNA chains have 5'-triphosphate termini and that the 5' terminal nucleosides are either adenosine or guanosine^{1–3}. Little is known about the fate of these termini. It is known that in *Escherichia coli* mRNA is degraded rapidly⁴, and that the nascent precursors of ribosomal and tRNA mature—a process involving trimming at the 5' end^{5–7}—and then have only one phosphate group on the 5' end^{7,8}. It seems, therefore, that the 5'-triphosphate termini should disappear as a result of mRNA decay and stable RNA maturation. We have examined the disappearance of triphosphate termini from *E. coli* RNA and found that they decay at a rate approximately one-half of that of the bulk of the unstable RNA.

We followed the decay of ³²P-labelled triphosphate termini in cell cultures after rifamycin SV had been added to stop further initiation of RNA synthesis⁹. Since *E. coli* is relatively resistant to rifamycin, we used the mutant strain *E. coli* H-101 (ref. 10), which is sensitive. To determine whether there was a lag in rifamycin action and how completely it blocked RNA synthesis, a control experiment on induction of the lactose operon was performed. This was similar to that done with strain AS 19 (ref. 11), except that adenosine-3',5'-cyclic monophosphate was added to avoid the effects of catabolite repression¹². Cells were induced with isopropyl-β-D-thiogalactopyranoside at various times before and after addition of rifamycin, protein synthesis was allowed to continue for 20 min, and

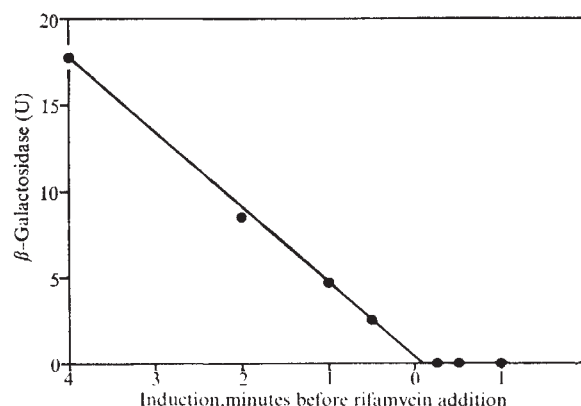


Fig. 1 Inhibition of induction of β-galactosidase synthesis by rifamycin SV. One ml of a growing culture of *E. coli* was added to each of 10 flasks containing 1 ml of 0.015 M adenosine-3',5'-cyclic monophosphate (neutralised to pH 7.0) and 0.2 ml of 6× growth medium. Cells were induced by addition of 0.3 ml of 0.01 M IPTG at various times before or after the addition of 0.6 ml of 500 μg ml⁻¹ of rifamycin SV. Twenty minutes after induction, cells were treated with toluene, and β-galactosidase was assayed by the method of Abbo and Pardee²⁰.

β -galactosidase activity was assayed (Fig. 1). The results of this control show that initiation of RNA synthesis was shut off within 15 s of the addition of $100 \mu\text{g ml}^{-1}$ of rifamycin.

Decay experiments were performed as follows. Cells, growing at 37°C , were labelled with ^{32}P -phosphate for 5 min, and rifamycin SV was added to stop further initiation of RNA synthesis. Culture samples were taken immediately after rifamycin addition and during the next 16 min. The samples were each mixed with a given volume of cells from a culture labelled with ^3H -guanine, to give an internal standard on RNA recovery in the purification steps. RNA was then isolated from the culture samples (see legend to Fig. 2) and purified on gel filtration columns. (This purification was necessary due to the accumulation of ^{32}P -labelled contaminants, presumed to be polyphosphates, which interfere with the detection of the pppNps.) The purified RNA samples were hydrolysed with KOH, releasing adenosine-2'(3')-monophosphate, 5'-triphosphate (pppAp), and guanosine-2'(3')-monophosphate, 5'-triphosphate (pppGp) from the triphosphate termini¹³. These compounds were identified by DEAE paper electrophoresis at pH 3.9 as already described¹³. ^{32}P was counted in a liquid scintillation counter and corrected to a reference date for radioactive decay.

We performed two experiments on the decay of triphosphate termini, differing in the purification of the RNA by gel filtration. In the first experiment, RNA was purified on a Bio-Gel P-10 column (exclusion approximately 20,000 daltons), retaining the excluded vol-

ume for analysis. The data show total ^{32}P c.p.m. in the RNA and in the triphosphate termini as mononucleotides and pppNps released by KOH hydrolysis of that RNA (Fig. 2). The second experiment was similar, except that RNA samples were divided in half, and each half was run on a separate Sephadex G-100 column. The eluates from these columns were separately pooled to include both the excluded peak and the material eluting up to a K_{av} (ref. 14) of 0.48. Calibration of similar columns, using single-stranded DNA fragments of known length, by Dr Donald A. Kaplan, indicates that this material includes oligomers of length approximately 12 and greater. The data for the second experiment shows ^{32}P c.p.m. in RNA, in total pppNps released by KOH hydrolysis, and also in pppAp with results from duplicate samples given to indicate the variability encountered in these experiments (Fig. 3). Further data, not given, show that the ratio of ^{32}P c.p.m. in pppGp to pppAp at the beginning of each experiment was 1.4 and that this did not change significantly throughout the experiments. Thus the kinetics for the decay of both guanosine and adenosine triphosphate termini were similar.

The data for the decay of RNA and triphosphate termini from these experiments can be approximated by exponential decay as seen by the straight lines drawn on the semi-logarithmic graphs. These approximations give half-lives of 3.4 and 6.0 min for the decay of the RNA and triphosphate termini respectively in the first experiment, and 2.1 and 4.6 min in the second experiment. Using a different method, Konrad estimated the half-life of the termini at 21.5° to be at least 170 s (ref. 15).

In interpreting these data, we assume either that the 5' ends of all species of nascent RNA are destroyed by the same mechanism or that the data reflect primarily the decay of a particular species of RNA, presumably the messenger. Preliminary work done in this laboratory by Charles Bieger, indicates that a significant proportion of the ^{32}P -labelled triphosphate termini on *E. coli* RNA after a 5 min pulse with ^{32}P -phosphate is found in polysomes; however, the exact amount depends on the method of extraction of the cells. If tRNAs were made monocistronically, one would expect their precursors to contribute the majority of triphosphate termini in the *E. coli* cell because these termini are a measure of the number and not the weight of RNA molecules¹³. There is, however, an indication that some tRNAs are made polycistronically⁶, and thus their contribution to the 5' termini will be reduced. Furthermore, as the nascent tRNA precursors are undetectable in normal cells growing at 37°C , their termini may disappear extremely rapidly⁶. Finally, the decay of triphosphate termini seen in these experiments could be the result of one of two processes. The 5' end of the RNA could be degraded so that the triphosphate termini would be on a small polynucleotide, for example, less than 12 nucleotides long in our second experiment, or by a process whereby one or more of the phosphates are removed from the triphosphate termini.

Our data rule out the possibility that the triphosphate termini decay much faster than does unstable RNA. In fact the decay of the former could be a result of the decay of the latter. Since the data indicate a longer half-life for the decay of triphosphate termini than for that of unstable RNA, they suggest that degradation of triphosphate termini occurs by a separate mechanism than that primarily responsible for the degradation of RNA.

From the results of work on the decay of both lactose mRNA and tryptophan mRNA, Kennell *et al.* concluded that each cistron in these polycistronic RNAs has a unique site that is vulnerable to attack, causing inactivation. This attack is presumably the initial event in the breakdown of the message^{4,16}. From work on the decay of tryptophan mRNA, Yanofsky *et al.* make a model for the

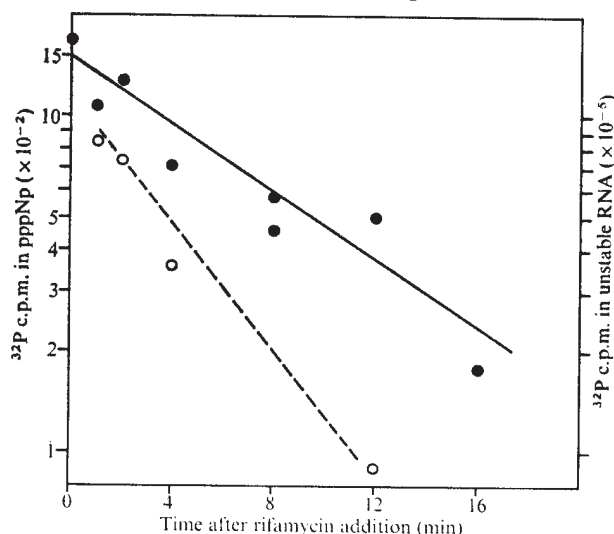


Fig. 2 Radioactivity in RNA (○) and in pppNps (●) after rifamycin treatment. Cells were grown at 37°C in glucose minimal medium³, using HEPES buffer and supplemented with $100 \mu\text{g ml}^{-1}$ of methionine. They were grown to an optical density (at 540 nm, 1 cm path) of 0.8, washed with low phosphate medium, shifted to low phosphate medium (2×10^{-5} M phosphate), incubated for 3 min, and pulse labelled with 50 mCi of ^{32}P -phosphate for 5 min. At the end of this labelling time, rifamycin SV and non-radioactive phosphate buffer (pH 6.8) were added to the culture to make final concentrations of $300 \mu\text{g ml}^{-1}$ rifamycin and 10^{-3} M phosphate. Culture samples were taken at various times and mixed with equal amounts of ^3H -guanine labelled cells. Cells were broken open in 2% sodium dodecyl sulphate at 60°C and RNA was isolated by phenol extraction, ethanol precipitation and purification on Bio-Gel P-10 (Biorad) gel filtration columns at 4°C . The excluded RNA fractions were then hydrolysed with KOH and the residual DNA and protein were precipitated with perchloric acid¹³. Samples were absorbed on charcoal and eluted with ethanol: 28% $\text{NH}_4\text{OH}:\text{H}_2\text{O}$ (66:2:32). The total ^{32}P counts in purified ribonucleotides were taken as total RNA and the ^{32}P counts in pppNps as total triphosphate termini (adjusted by ^3H -guanine nucleotide recovery). The data for the decay of RNA were corrected by the subtraction of the stable RNA fraction (that remaining after 16 min).

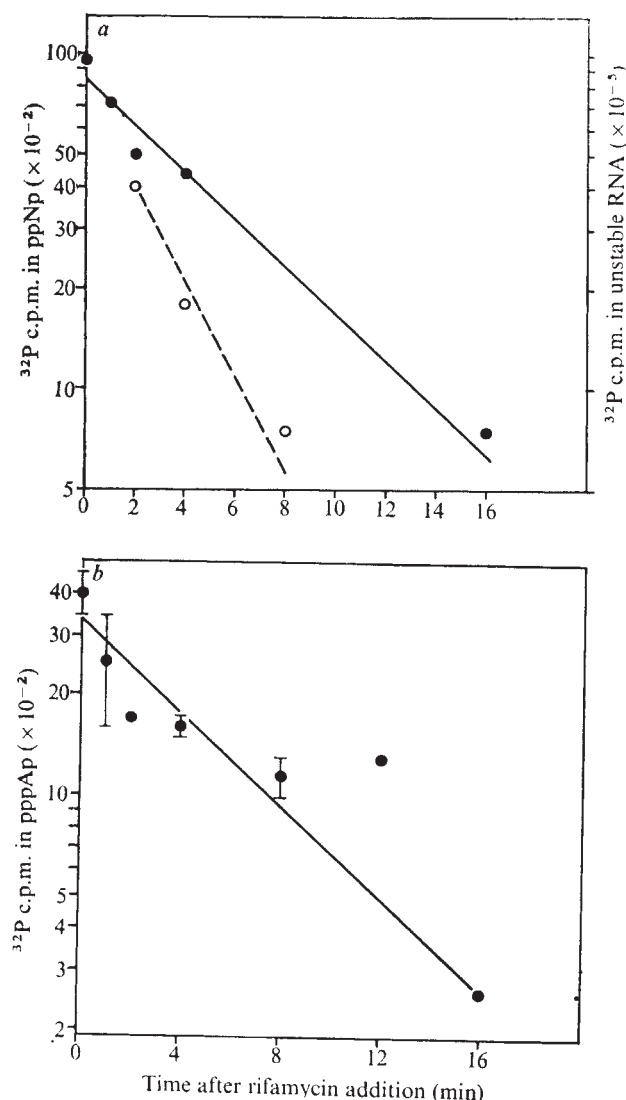


Fig. 3 Radioactivity in RNA (○) and in pppNps (●) after rifamycin treatment. The experiment was performed as described for Fig. 2, except that the RNA was purified on Sephadex G-100 (Pharmacia) columns as described in the text. a, ^{32}P c.p.m. in RNA and in total pppNp; b, ^{32}P c.p.m. in pppAp. Upper and lower brackets show data from duplicate columns.

decay of mRNA in which the initial degradative event occurs near the 5' end of the molecule and is followed by degradation in the 3' direction¹⁷. We favour a model where internal degradation is the first step in mRNA decay. Such a model is supported by the discovery of 5'-terminal fragments of the transcripts of the T7 early-genes and tryptophan operon^{18,19}. The longer half life of the 5' terminus could be the result of the presence of the triphosphate termini inhibiting a 5'-exonuclease attack. Alternatively, if messenger degradation is associated with translation, the 5' end of the molecule, which is not translated, would be degraded by a separate mechanism.

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Model for wandering restriction enzymes

I wish to propose a model for those DNA restriction enzymes which, although requiring specific recognition sites, make a limited number of cuts at apparently random sites, far from the recognition sites.

Many bacteria have restriction and modification enzymes which recognise and attack DNA from other organisms (for recent reviews see refs 1, 2 and 3). A modification enzyme recognises a particular nucleotide sequence and modifies it, for example, by methylating a nucleotide in this sequence. DNA from other organisms, lacking this modification, is recognised as foreign, and is cut by a restriction enzyme. On the other hand, DNA which has been modified or which does not include the recognition sequence is not cut, that is, it is not restricted. Both strands of DNA are usually modified. Replication of modified DNA yields DNA of which one strand (the parental) is modified, and the other (the daughter) is unmodified. Modification in only one strand prevents restriction but permits modification of the other strand. In this way DNA can replicate without becoming sensitive to restriction.

Restriction enzymes fit into two groups on the basis of their restriction products. Enzymes of group I, such as those of *Haemophilus*⁴ and those encoded by the RI⁵ and RII⁶ resistance transfer factors, cut at unique sites of the DNA yielding a population of specific fragments⁷⁻⁹ with unique terminal sequences. The terminal sequences, where known, imply that the nucleases act at symmetric DNA nucleotide sequences⁴⁻⁶, that is sequences such as



which reads the same from left to right as from right to left. This sequence, a substrate of a *Haemophilus* enzyme⁴, consists of two identical components (pGpTpTp / pCpApAp) arranged symmetrically.

Within a symmetric sequence both strands look alike. A modification or restriction enzyme which recognises part or all of the sequence could act on both strands, either sequentially, first on one strand and then on the other, or, if the enzyme were a symmetric dimer, simultaneously on both strands.

Restriction enzymes of group II, those of *Escherichia coli*¹⁰⁻¹² and phage P1 (unpublished results of K. Horiuchi, quoted in ref. 13), recognise specific sites, but in contrast to group I enzymes they make a limited number of double strand cuts at

apparently random sites, far from the recognition sites. For example, the circular DNA molecule that is the replicative form of phage f1 is cut only once by the *E. coli* B restriction enzyme, yielding a linear molecule which is resistant to further degradation. The cuts generate a heterogeneous population of molecules which can be denatured and renatured to reform circular molecules. These circular molecules, in contrast to the linear molecules, are again sensitive to the restriction nuclease, indicating (1) that the recognition sites are not inactivated by the first cut, and (2) that a circular molecule is necessary for the recognition sites to be effective¹¹.

A puzzling feature is the difference between the number of cuts introduced and the number of recognition sites. Phage f1 has two widely separated recognition sites (denoted sB_1 and sB_2) which render the phage DNA sensitive to the *E. coli* B restriction nuclease¹⁴. *In vivo*, phage bearing mutations in either sB_1 or sB_2 are partially resistant to restriction, and phage carrying both mutations are completely resistant. Although the presence of either recognition site permits a cut by the restriction nuclease, the two sites together do not permit two cuts¹¹.

Figure 1 depicts a model for the action of group II restriction nucleases. Suppose that a specific asymmetric (unmodified) recognition sequence permits binding of the nuclease^{12,15}. Having bound, the enzyme now wanders (in the direction defined by the orientation of the recognition site) along the DNA until it meets a nuclease which started from an oppositely oriented sequence. A meeting, head to head, is the signal to cut the DNA. The cutting might occur at many different sites, depending on where the nucleases meet. Once the first double strand cut is introduced, enzymes starting from these recognition sites would no longer be able to meet, accounting for the resistance of the linear molecule to further cutting.

In this model the complete recognition site might be viewed as comprising two identical nucleotide sequences arranged in opposite orientations. The stretch of DNA between the two components of the recognition site might be thousands of

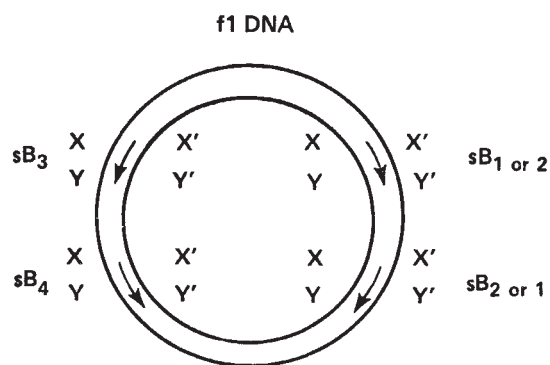


Fig. 2 Proposed arrangement on phage f1 DNA of the recognition sites of the restriction modification system of *E. coli* B.

nucleotides (the case of phage f1)¹¹ or only a few nucleotides in which case the restriction enzyme would seem to act like a group I enzyme.

The complete recognition site, composed of two oppositely oriented identical sequences is symmetric. Whereas for group I enzymes, the two components of the symmetric recognition region are juxtaposed, for group II enzymes the two components can be separated by a long and variable nucleotide sequence, and the nuclease can act anywhere in this nonspecific sequence.

Modification (methylation) of the DNA of phage fd (perhaps identical to phage f1) is blocked by mutations at sites sB_1 and sB_2 ¹⁶, suggesting according to this model, that modification of the putative sites sB_3 and sB_4 depends on the sites sB_1 and sB_2 . The modification enzyme might bind at the recognition site of one strand and wander to a recognition site in the other strand, which it would then modify, thus blocking the binding of restriction enzyme and protecting the DNA. Two oppositely oriented recognition sites would be required for modification activity, as for restriction activity. The type of interaction between the modification enzyme and recognition site would depend on how each component is presented. Free enzyme would bind to but not modify a recognition site. Wandering enzyme would modify a recognition site presented in the orientation opposite the orientation of the site to which that modification enzyme had bound.

The restriction nuclease might also interact differently with the two components of the recognition site. According to the version presented in Fig. 1, the restriction nuclease might bind at one recognition site and wander past the complementary site, finally encountering the complementary nuclease in the region S'. Cutting in the region S' should not preclude cutting in the region S. Only one cut is observed, however, for f1 DNA, suggesting that the restriction nuclease cannot pass a recognition site, perhaps because of the particular experimental conditions used, or perhaps because wandering restriction nuclease specifically interacts with a recognition site to obviate further wandering.

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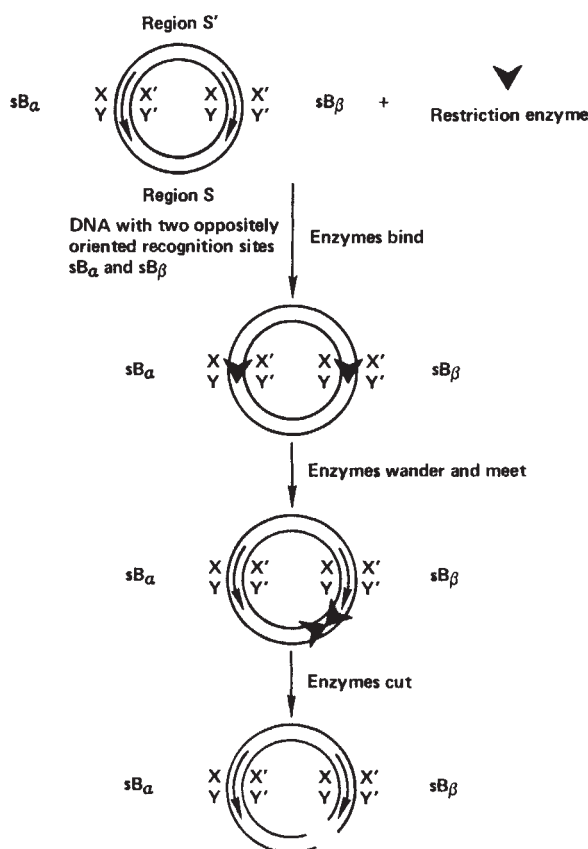


Fig. 1 Model proposed for group II restriction enzymes.

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C-type viruses in systemic lupus erythematosus

VIRUSES have been implicated as the agents of systemic lupus erythematosus (SLE) expressed by dogs¹ and mice². We have shown that cell-free filtrates prepared from the spleens of dogs with positive LE cell tests induced anti-nuclear and anti-double-stranded DNA antibodies in CAF₁ mice. The canine filtrate also induced lymphoid tumours in some recipient mice¹. One of these neoplasms, SP 104, produces a monoclonal antibody against double-stranded DNA. Cultured SP 104 cells produce, in addition to the anti-DNA antibody, C-type RNA viruses that induce anti-nuclear antibodies when inoculated into normal mice (R.M.L., W.T., C.S., and R.S.S., unpublished). Here we present preliminary evidence linking the SP 104 virus to SLE in humans. Our results suggest that antigenic determinants related to the SP 104 virus are present on the lymphocytes of patients with SLE.

An antibody to the SP 104 virus was made in rabbits using purified C particles that were isolated from the fluid of cultured SP 104 cells by isopycnic centrifugation³ through a 15–60% sucrose gradient. The virus had a buoyant density of 1.16 and all the reverse transcriptase activity of the preparation was localised to this band of the gradient. The banded virus contained 70S RNA, behaved as a B-tropic MuLV in the XC plaque assay, contained the gs-1 antigen of MuLV, had a PI of 3.2 by isoelectric focusing and by electron microscopy consisted almost entirely of C-type particles.

The purified virus was disrupted with ether⁴ and injected into New Zealand rabbits. Ten days after two subcutaneous injections of the virions, serum was collected and absorbed sequentially with foetal calf serum, normal murine (CAF₁) spleen cells and blood lymphocytes from normal human subjects. By gel diffusion analysis, a single line of identity was obtained when purified undisrupted and ether-treated SP 104 virions were reacted with the absorbed serum. The absorbed serum failed to react with DNA in gel diffusion tests and did not bind to ³H-labelled DNA in a sensitive radioimmunoassay for anti-DNA antibodies⁵. Neither antinuclear antibodies nor antibodies against human or murine serum proteins or cell membrane were detectable in the rabbit serum.

A globulin-rich fraction of the absorbed serum was prepared by ammonium sulphate precipitation⁶. The final product reacted with SP 104 virions in gel diffusion and failed to react in any of the above mentioned tests. The

globulins were then conjugated to fluorescein isothiocyanate in a molar ratio (fluorescein:protein) of 10:1 (ref. 7). Lymphocytes, separated from the blood of subjects by centrifugation through a Ficoll-Hypaque gradient⁸ and further purified by removal of phagocytes⁹, were incubated with the conjugate, washed, fixed in ethanol and examined by dark field fluorescence microscopy. The lymphocytes of normal subjects failed to react with the conjugate. By contrast, typical and brilliant membrane fluorescence was observed on the lymphocytes of five out of six patients with SLE. The number of lymphocytes stained by the conjugate ranged from 46×10^5 to 400×10^5 cells. Individual values for the patients studied were 0, 46, 149, 181, 195, and 400. These figures were obtained by examining at least 200 cover slip fields, each of which contained between 400 and 800 lymphocytes. Independent experiments performed on the same cell preparations by two of us gave results that agreed within a margin of 20%. Normal rabbit globulins, treated in the same way, failed to react with any of the lymphocytes.

We next sought evidence in a patient with SLE of an antibody with reactivity similar to that of the rabbit anti-SP 104 virus antibody. A 12-yr-old girl (DeV) was selected as a subject because she had active, untreated SLE with multi-system involvement. Her serum was treated with ammonium sulphate, conjugated to fluorescein isothiocyanate, and used to stain the blood lymphocytes of various subjects in the same manner as the rabbit serum. DeV's serum failed to bind to the cytoplasmic membranes of lymphocytes from either six normal subjects or from six patients with diseases other than SLE. By contrast, typical membrane staining was found with the lymphocytes of eight out of ten patients with SLE (Table 1). The number of lymphocytes stained by the conjugate ranged from 6×10^5 to 650×10^5 lymphocytes, a proportion similar to that observed with the rabbit antiserum. Raising the temperature of the reaction mixture to 37° C for 30 min (following incubation at 4° C) resulted in polar capping of the fluorescent stain. The reactivity of the DeV reagent was abolished by previous incubation with a monospecific rabbit anti-human IgG serum, but not by incubation with a rabbit anti-human fibrinogen serum. Immunoglobulins prepared in the same way from normal human serum did not bind to any of the lymphocytes of five patients with SLE.

The immunoglobulin fraction of DeV's serum was absorbed with pelleted C-type particles obtained from the SP 104 culture. The absorbed material, after conjugation to fluorescein isothiocyanate, failed to stain the lymphocytes of five out of eight patients with SLE. In a seventh patient (T.O.U.), absorption of DeV's serum with C-type particles reduced the reaction by about 90%. Absorption of the DeV conjugate with a suspension of 600×10^6 normal murine spleen cells failed to diminish its reaction with lymphocytes of patients with SLE. Moreover, the DeV conjugate failed to stain the cytoplasmic membranes of normal murine spleen cells. Removal of all anti-DNA antibodies by absorption of the conjugate with double- and single-stranded DNA also failed to diminish its ability to bind SLE lymphocytes, except in two cases, which are discussed presently. In a preliminary experiment, we also found that absorption with yeast (single-stranded) RNA and poly(I)-poly(C) (double-stranded RNA) also failed to remove the activity of DeV's serum against SLE lymphocytes.

Three of the eight patients we tested (L.E.V., F.A.Z., and T.O.U.) differ from the others in that they were clinically ill when their lymphocytes were examined; that relatively large numbers of their lymphocytes reacted with the DeV conjugate, the anti-SP 104 virus serum, or both; that absorption of this reactivity by the SP 104 virus was incomplete and that absorption by DNA removed some of the activity (F.A.Z., and T.O.U.). These results indicate that more than one SLE-related antigen may

Table 1 Fluorescent lymphocytes per 10⁶ cells

SLE Patient	DeV conjugate	Absorption with:		
		SP 104	D-DNA	S-DNA
C.R.O.	0	—	—	—
H.E.A.	0	—	—	—
B.O.U.	6	0	44	30
H.I.E.	20	0	13	30
H.U.N.	92	0	166	—
C.O.O.	61	0	—	—
R.U.G.	36	0	32	—
L.E.V.	356	187	275	173
F.A.Z.	350	202	256	62
T.O.U.	650	44	410	76

SP 104, the pellet containing C-type virus particles; D-DNA, double-stranded DNA; S-DNA, single-stranded DNA.

appear on the membranes of circulating lymphocytes during the acute phase of the illness.

The antibody we detected in DeV's serum is not the anti-lymphocyte antibody described in SLE (ref. 9), nor is it an anti-IgG that stains B cells¹⁰ since it does not react with normal lymphocytes. We have tried to stain PHA-transformed lymphocytes from normal subjects with the DeV reagent, but no reaction was detected. The activity is not due to an antibody against the Epstein-Barr virus as no anti-EBV activity in DeV's serum has been found. Moreover, the determinant on the test lymphocyte is not an anti-IgG that might bind to the DeV reagent⁵ because conjugated normal IgG did not react with the cells. We do not know if DeV's serum is unique; tests with the sera of other patients with SLE are in progress.

It seems that the lymphocytes of patients with SLE express an antigenic determinant shared by C-type RNA viruses produced by the SP 104 tumour. This plasmacytoma, we stress, also produces an antibody against double-stranded DNA; such antibodies are characteristic of SLE. Experiments are now under way to determine if the SP 104 virus is unique in its relationship to SLE. Our results do not exclude a virus-induced cellular antigen. Results with the rabbit antibody, however, which is directed against purified virus, suggest the presence of a virion determinant on the cytoplasmic membranes of lymphocytes of patients with SLE.

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Rapid *in vivo* assay for murine lymphatic leukaemia viruses

SEVERAL rapid *in vitro* methods¹⁻⁵ for the quantitative assay of murine lymphatic leukaemia viruses (MuLV) have replaced the once standard *in vivo* bioassay for leukaemogenicity^{6,7}, which was slow, cumbersome, and relatively costly. The *in vitro* procedures, however, suffer from two limitations: first, some MuLV propagated serially *in vitro* may lose leukaemogenicity while retaining infectivity *in vitro*⁸, so the *in vitro* assays do not necessarily correlate with biological activity *in vivo*; second, some wild-type MuLV extracted from normal or neoplastic tissues *in vivo* seem to require a period of adaptation to *in vitro* growth conditions before they can be assayed reliably by *in vitro* methods⁹.

Both of these limitations apply to the radiation leukaemia virus (RadLV), a naturally occurring MuLV consistently extractable from thymic lymphomas induced in strain C57BL mice by whole-body X-irradiation, which induces thymic lymphomas after inoculation into neonatal or infant C57BL/Ka mice (*in vivo*-derived wild-type RadLV)^{6,10}. It can also be propagated on mouse embryo fibroblasts⁸ and collected from the supernatant of permanently infected cultures (*in vitro*-derived RadLV, designated RadLV*). Several of the rapid *in vitro* assay methods have recently been adapted to the titration of RadLV* (refs 9, 11 and 12 and A.D., O.N., E. Gerlmann, and H.S.K., unpublished). Here we report a rapid *in vivo* endpoint dilution assay for wild-type RadLV which compares in sensitivity with *in vitro* endpoint dilution methods, correlates well with leukaemogenicity bioassays, and should be equally applicable to the *in vivo* assay of other thymotropic MuLV in their susceptible host strains.

C57BL/Ka mice of both sexes were used at 4–5 weeks of age. Wild-type preparations of RadLV were extracted from RadLV-induced lymphoma tissue by a previously described procedure¹³. Serial five-fold dilutions of virus in medium containing Polybrene (Abbott Laboratories), 4 µg ml⁻¹, were injected in a volume of about 0.02 ml per lobe into one or both thymic lobes of groups of test mice¹⁴. A group of mice was also injected intravenously with 0.2 ml of serially diluted virus. Thymus cell suspensions were prepared at serial time intervals thereafter. Mice were killed with ether. The thymus was dissected free, minced in a small Petri dish, and passed through nylon cloth into (0.175 M NaCl) saline. Cells were kept in suspension on ice, and their concentration was adjusted to 1 × 10⁶ cells ml⁻¹.

Indirect immunofluorescence (IF) staining of acetone-fixed cells for the detection of MuLV antigens was carried out as described by Hilgers *et al.*⁴ and adapted to virus titration

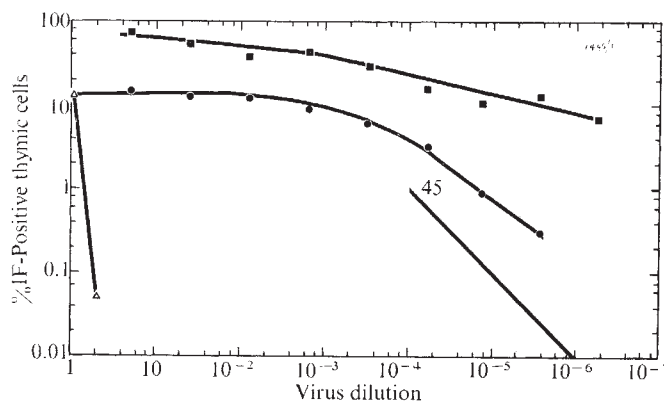


Fig. 1 Titration curves for serial dilutions of RadLV injected intrathymically into one (●) or both (■) lobes of the thymus, or injected intravenously (△). A 45° slope curve is included for comparison.

Table 1 % IF-positive thymic cells per mouse after direct virus injection into one lobe of the thymus

Time after inoculation	5 ⁰	5 ⁻¹	5 ⁻²	5 ⁻³	Virus dilutions		5 ⁻⁶	5 ⁻⁷	5 ⁻⁸	5 ⁻⁹
					5 ⁻⁴	5 ⁻⁵				
1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	NT	NT
4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	NT	NT
7	15,16	11,12, 14,15	10,12, 13,15	4,7,10,14	1,7,8,9	2,3,3,4	0.7,1.0, 1.0,1.0	0.0, 0.0, 0.5,0.7	NT	NT
14	66,69	74,82, 84,90	50,74 86,92	73,74, 90,96	60,65, 90,92	25,51 54,69	23,29, 45,64	0.0,7.5, 9.0,63	0,0,0,60	0,0
21	70,72	72,80, 85,90	79,83	70,77	70,80	69,78	65,72	0,78,87	0,0,0,0	0,0,0,0
28	NT*	NT	NT	NT	NT	NT	NT	60,80	0,90,90	0,0,0,0
Total no. mice tested	10	16	14	14	14	14	14	17	11	10
No. positive after 14 or more days	4/4	8/8	6/6	6/6	6/6	6/6	6/6	7/9	3/11	0/10
No. tested										

* Not tested.

in vitro by Declève *et al.*⁹. The percentage of IF-positive cells recorded in each assay was that observed at the highest concentration of rat anti-serum yielding fluorescence specific for virus-related antigens^{4,9}. Counts of 100–200 total cells suffice when IF-positive cells are abundant; conversely, fields containing 20,000 or more cells may be scanned to detect low frequencies of IF-positive cells. Direct examination of thymic lymphoid tissue for viral antigens by immunofluorescence was found to be straightforward, since no nonspecific reaction with immunoglobulin-bearing lymphoid cells could be detected in the normal rat serum or saline controls.

After direct intrathymic (IT) injection of RadLV into one thymic lobe, virus-related antigens were first detected at 7 d for RadLV dilutions ranging from 5⁰ to 5⁻⁷ (Table 1). At 14 d after inoculation, 3 of 11 mice were positive at the 5⁻⁸ dilution, and 0 of 10 at the 5⁻⁹ dilution, yielding an endpoint titre of 3.9×10^6 per 0.02 ml. When the percentage of IF-positive cells observed on day 7 after inoculation is plotted against virus dilution on a log-log scale (Fig. 1), a plateau value of 12–15% for dilutions 5⁰–5⁻³ is seen, followed by a decrease in percentage IF with increasing virus dilution. The slope of the curve is less than 45°.

When both lobes of the thymus were injected with 0.02 ml each of RadLV, virus-related antigens were detected as early as 2 d after inoculation of high concentrations of virus. The percentage of positive cells in all positive mice at one week was above 2%; among the negative mice, no positive cells were found among more than 20,000 examined. The endpoint titre for the 5⁻⁹ virus dilution was detected by 7 d (endpoint titre 1.9×10^6 per 0.04 ml). The curve obtained when the results were plotted on a log-log scale (Fig. 1) again has a slope much less than 45°, thus ruling out the interpretation, based on the results of unilobar injection, that the reduced slope might be due to selective virus-induced involution of the injected lobe¹⁶. Instead, the slope of less than 45° is apparently due to secondary infection; accordingly, the assay yields valid results only as an endpoint dilution procedure.

In previous studies using the leukaemia induction bioassay^{6,7}, it was shown that the intravenous (IV) route of viral injection is about 100 times less sensitive than the IT route. The same difference in sensitivity has now been observed with the new IF assay. At 3 week after IV injection of serial RadLV dilutions, the IF endpoint titre was 3.1×10^3 , about 100 to 1,000 times less than that obtained after intrathymic injection.

Up to now the bioassay method^{6,7} has been the only assay by which one could determine how many leukaemogenic particles were present in a given volume of RadLV preparation. To obtain a direct comparison of the two *in vivo* assays, the same stock of virus used for the unilobar IF assay described above was inoculated IT (0.02 ml) into one thymic lobe of C57BL mice. The endpoint was scored after 9 months

by determining the highest dilution of RadLV which induced lymphomas. The endpoint dilution was 5⁻⁸ (titre 3.9×10^6), in excellent agreement with the titre obtained after unilobar injection in the *in vivo* percentage IF assay. Several replicate *in vivo* IF assays, using IT injection, have indicated good reproducibility of the method.

The estimated titres obtained by three *in vitro* assay methods are as follows: the reverse XC cell test¹¹, the percentage IF assay⁹, and the UCI-B focus assay^{5,13} were comparable (1.9×10^6 per 0.4 ml) to those obtained with the new *in vivo* percentage IF assay. The direct XC test³, however, and the indirect helper focus assay¹² gave lower (1×10^5 per 0.4 ml) and higher (9.6×10^8 per 0.4 ml) titres, respectively. *In vivo*-derived virus preparations have been more difficult to assay by rapid *in vitro* methods because major differences exist in viral growth kinetics between the *in vitro*-derived and *in vivo*-derived preparations of RadLV.

The new *in vivo* assay is direct, rapid, and sensitive. The highest dilution of RadLV capable of inducing a lymphoma is also the highest dilution capable of infection and active virus replication in thymus cells, as revealed by the appearance of virus-related antigens in the cell cytoplasm. The assay should therefore prove useful in elucidating the sequence of events *in vivo* between the time of virus inoculation and the ultimate development of lymphomas induced by this and other thymotropic MuLV.

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Table 1 Mixtures of *k* anti-*d* killing cells and normal or *d* anti-*k* added cells: percentage of surviving cells and residual cytotoxicity for *d* target cells

Exp.	Added cells*	Cytotoxicity† for <i>d</i> target cells			% surviving cells‡		
		Ratios of added to killing cells			Ratios of added to killing cells		
		0:1§	0.5:1	1:1	0:1	0.5:1	1:1
1	normal <i>d</i>	54	65	60	80	Not done	Not done
	<i>d</i> anti- <i>k</i>		36	9			
2	normal <i>d</i>	46	52	48	70	43	35
	<i>d</i> anti- <i>k</i>		6	1		30	25
3	normal <i>d</i>	54	60	52	55	27	30
	<i>d</i> anti- <i>k</i>		14	2		45	20

C3H anti-BALB/c (*k* anti-*d*) and BALB/c anti-C3H (*d* anti-*k*) sensitised cells were obtained after 5 d of coinubation at 37°C in a 10% CO₂ atmosphere of 7 × 10⁶ responding spleen cells and 7 × 10⁶ irradiated (2,000 rad) stimulating spleen cells in 2.5 ml of modified Eagle medium plus 10% heat-inactivated foetal calf serum, in the presence of 10⁻⁵ M mercaptoethanol^{27,28}, per well of FB-16-24-TC Linbro plates.

*Added cells were either normal BALB/c (*d*) spleen cells or sensitised *d* anti-*k* cells. They were transferred into type 2003 plastic Falcon tubes, in 1 ml of medium plus serum, with 2 × 10⁶ *k* anti-*d* sensitised killing cells, at the indicated ratios of added cells to killing cells. After one centrifugation at 180g for 5 min, the tubes were incubated overnight at 37°C and centrifuged again. The supernatants were pipetted out, 1 ml of fresh medium plus serum was added per tube and the resuspended cells were tested for their cytotoxicity and counted in a trypan blue exclusion test.

†Cytotoxicity was measured as follows. Target cells were P815 mastocytoma cells (*d*), labelled overnight with 100 µCi of ⁵¹Cr in 1–2 ml as previously described⁶, washed twice and distributed in V-shaped wells of IS-MVC-96 Linbro microplates (10⁴ P815 cells per 0.1 ml per well). Each well also received 0.1 ml of one of the cell suspensions tested for their cytotoxicity. The microplates were centrifuged (280g, 2 min) to increase the sensitivity of the cytotoxicity test^{6,29}, incubated for 4 h at 37°C in a humidified CO₂ atmosphere and centrifuged again. Volumes of 0.1 ml of supernatants were sampled with an Eppendorf micropipette, their radioactivity was measured and expressed as % radioactivity initially present in 10⁴ P815 target cells. Cytotoxicity, in this table, is expressed as % ⁵¹Cr-release averaged from triplicate wells, minus 10% which was spontaneous ⁵¹Cr-release by target cells alone in each of these three experiments. The cytotoxicity for *d* target cells of *d* anti-*k* added cells alone was, for experiments one to three, 3, 3, and 2, respectively. This shows the degree of specificity of these anti-*k* cytotoxic cells. In this type of assay, an experimental difference of 5% or more is statistically significant (*P* = 0.05)¹¹.

‡Percentage of trypan blue-excluding cells after overnight incubation, compared to the initial number of trypan blue-excluding cells in the mixtures of added cells and killing cells. These figures can be used to determine the experimental parameters not given in the table. For instance, for experiment three, column 0.5:1, the initial number of cells was 10⁶ added normal cells plus 2 × 10⁶ killing cells, thus 3 × 10⁶ cells. The percentage of surviving cells was 27%, the number of surviving cells was thus 8 × 10⁵. One tenth of this was used for the cytotoxicity test, giving a ratio to target cells of 8:1. The corresponding cytotoxicity was 60.

§Killing cells incubated alone, without added cells.

Let us provisionally make the hypothesis of a nonspecific cytotoxic factor, released into the extracellular fluid by the killing cell upon specific contact with the relevant target cell, which would be necessary and sufficient for subsequent target cell lysis. This extracellular cytotoxic factor would lyse the target cell, and should eventually lyse the cytotoxic cell as well. The cytotoxic cell is not lysed, however, either during or after the killing process^{24,25}, and is able to kill other target cells^{2,9,26}. The cytotoxic cell must then be resistant to the factor. If the cytotoxic cell (A) is subjected to the effect of other cytotoxic cells (anti-A), the anti-A cells would, by hypothesis, act against A cells through the release of a soluble factor. A cells, being resistant to the factor, must be resistant to the effect of anti-A cells as well. In other words, our initial hypothesis about a

Sensitivity of cytotoxic T cells to T-cell mediated cytotoxicity

LYMPHOID cells sensitised against alloantigens can lyse *in vitro* target cells bearing these alloantigens^{1,2}. In most of the experimental systems using *in vivo* sensitised cells, T cells (thymus-dependent lymphocytes) are both necessary³ and sufficient^{4,5} for induction of target cell lysis. This seems to apply also to *in vitro* sensitised cells^{6–9}. Specific cytolysis can be described as a two stage process: first, the recognition of target cell antigens by specific receptors at the surface of the 'killing' cell^{10,11}; and, second, lysis itself.

Speculations about the mechanism of the second stage vary between two extreme hypotheses, that is, target cell lysis being exclusively due, either to direct membrane-membrane interaction with the killing cell, or to the effect of a nonspecific cytotoxic factor, a 'lymphotoxin', released in the surrounding fluid by the killing cell. Direct search for this or similar factors provided contradictory results^{12–23}. To investigate the possibility of a lymphotoxin involvement, I resorted to a different approach.

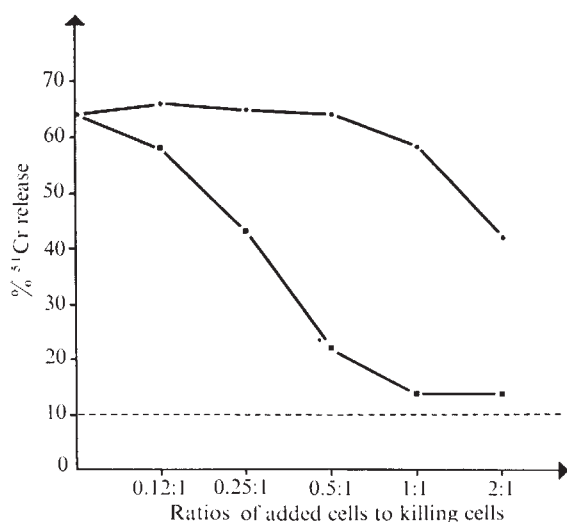


Fig. 1 Cytotoxicity, expressed as ⁵¹Cr release, of mixtures of *k* anti-*d* killing cells and added cells. A constant amount of killing cells (2 × 10⁶) was incubated overnight in the presence of various amounts of added cells, either normal *d* cells (●) or *d* anti-*k* cells (■). The surviving cells were tested for their cytotoxicity against *d* target cells, as indicated in the legend of Table 1. — — —, Spontaneous release by target cells alone.

soluble factor leads to the prediction that cytotoxic cells should be resistant to other cytotoxic cells. In fact, I found that cytotoxic T cells are sensitive to the effect of other cytotoxic T cells specifically directed against them. This sensitivity, contrasting with the survival of the cytotoxic cell when it kills a target cell, argues strongly against an 'all lymphotoxin' mechanism accounting for the lytic event.

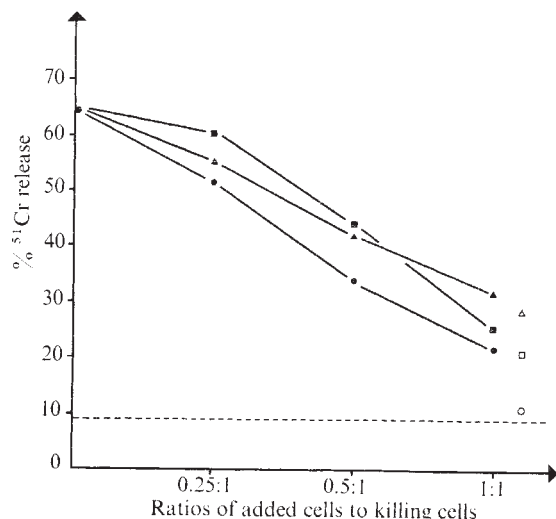


Fig. 2 Cytotoxicity, expressed as ^{51}Cr release, of mixtures of $b \times k$ anti- d killing cells and added cells. A constant amount of killing cells (2×10^6) was incubated overnight in the presence of various amounts of added cells, either b anti- k (■), or k anti- b (▲), or d anti- k (●). The surviving cells were tested for their cytotoxicity against d target cells. Δ , $\square$ and $\circ$ indicate cytotoxicity of added cells alone; — — —, spontaneous release by target cells alone.

Table 2 Mixtures of $b \times k$ anti- d killing cells and either no or normal or sensitised added cells: percentage of surviving cells and residual cytotoxicity for d target cells

Exp.	Added cells	Cytotoxicity for d target cells				% surviving cells			
		Ratios of killing to added cells				Ratios of killing to added cells			
		0.5:1	1:1	2:1	4:1	0.5:1	1:1	2:1	4:1
1	none*	31	44	53	57	80	70	70	78
	normal b	34	48	55	58	53	75	73	52
	b anti- k	6	10	25	48	40	30	30	44
2	none	20	33	43	52	40	60	55	55
	normal b	21	36	46	52	40	35	33	48
	b anti- k	5	12	29	45	27	30	37	48
3	none	41	53	59	60	80	80	50	35
	normal k	53	60	66	64	47	40	43	46
	k anti- b	11	36	58	65	20	20	30	24
4	none	25	40	45	45	80	60	70	55
	normal k	39	45	50	50	47	40	40	48
	k anti- b	8	26	45	47	27	20	33	46

Experimental protocol similar to the one given in legend of Table 1, except that a constant amount of added cells (10^6) is incubated overnight with varying amounts of killing cells. Killing cells were (C57BL/6 $\times$ CBA) F_1 hybrid anti-BALB/c cells. Added cells were either normal C57BL/6, or normal AKR, or C57BL/6 anti-AKR, or AKR anti-C57BL/6 cells. For experiments one to four the spontaneous ^{51}Cr -release by target cells alone was 9, 10, 12 and 8, respectively, and the cytotoxicity above spontaneous release of sensitised added cells alone was 5, 2, 10 and 8, respectively, which shows the degree of cross-reactivity of k anti- b cells in particular towards d target cells.

*Added cells were not included. In these experimental groups the amounts of killing cells were the same as in the corresponding groups with added cells.

In a first set of experiments, C3H anti-BALB/c (H-2 k anti H-2 d ; k anti- d for short) killing cells were incubated overnight together with either d anti- k or normal d 'added' cells. The residual anti- d cytotoxic activity of the mixtures in three consecutive experiments is given in Table 1. Cytotoxicity is much more depressed when d anti- k cells are added instead of normal d cells. An additional experiment showed (Fig. 1) that eight times more normal cells were necessary to depress cytotoxicity to the same extent as anti- k cells. The difference between both types of added cells was significant even at a ratio as low as 0.12 added cell to one killing cell, corresponding in this experiment to a final ratio of less than one added cell to one target cell. These results would be consistent with the possibility that the k anti- d killing cells are lysed or inactivated by the anti- k added cells, but other types of cell interactions, such as mechanical blocking³⁰, booster or 'interkill' effects, may also be involved.

In a second set of experiments, I therefore used another strain combination. (C57BL/6 $\times$ CBA) F_1 hybrid anti-BALB/c killing cells ($b \times k$ anti- d) were incubated overnight together with either d anti- k , or b anti- k , or k anti- b added cells. Use of the latter two types of added cells eliminates the possibility of blocking or interkill. A profound anti-cytotoxic cell effect of added cells was readily obtained in various experimental situations, for example when a constant amount of killing cells was preincubated with various amounts of added cells (Fig. 2), or when a constant amount of added cells was preincubated

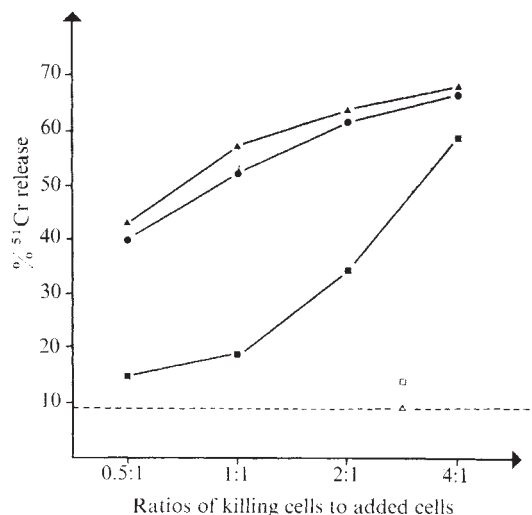


Fig. 3 Cytotoxicity, expressed as ^{51}Cr release, of mixtures of $b \times k$ anti- d killing cells and added cells. Various amounts of killing cells were incubated overnight either alone (●) or in the presence of a constant amount of added cells (10^6). Added cells were either normal b cells (▲) or b anti- k cells (■). $\square$ and Δ indicate cytotoxicity of added cells alone. — — —, Spontaneous release of target cells alone. More complete data for this experiment are given in Table 2, experiment 1.

with various amounts of killing cells (Fig. 3 and Table 2). Clearly, incubation of $b \times k$ anti- d killing cells with either k anti- b or b anti- k added cells led to a much lower residual anti- d cytotoxic efficiency than incubation with normal added cells. Finally experiments with anti- θ_{AKR} antiserum showed that the effect of k anti- b added cells on $b \times k$ anti- d killing cells could be abolished by pretreatment of the added cells with anti- θ antiserum plus complement, but not with anti- θ antiserum or complement alone.

Addition to killing cells of cells specifically sensitised against them leads to a decrease of the cytotoxic efficiency of the killing cells. This is consistent with the possibility that cytotoxic

cells can be lysed by other cytotoxic cells. These cytotoxic cells may not be lysed, however, but merely functionally inactivated. Some degree of cell lysis definitely occurred in mixtures of killing cells and sensitised added cells, in which the number of surviving cells was usually lower than in mixtures of killing cells and normal added cells. In any case, even functional inactivation of killing cells without lysis, and *a fortiori* lysis itself, mean that killing cells are sensitive to the effect of other cytotoxic T cells directed against them.

This result is contrary to the prediction derived from my initial hypothesis of a lymphotoxin-mediated lysis. Complete rejection of this hypothesis, however, would be founded on the formal line of reasoning given above, which may be endangered by any parameter not included in its premises (for instance, resistance of cytotoxic cells limited to the short time when they actually kill, or existence of a different mechanism for lysis of tumour target cells and lymphoid cytotoxic cells). Still, the present report makes it much less likely that the lytic phase of specific T-cell mediated cytotoxicity is an 'all lymphotoxin' one, that is, one entirely accounted for by a nonspecific extracellular soluble factor. This does not exclude a role for soluble factors, which may still be involved, for instance if directly injected by the killing cell into the target cell, or as an extracellular agent completing membrane-induced lesions. Alternatively, no soluble factors at all may be involved, cytolysis being exclusively due to membrane-membrane interactions. Whatever the lytic mechanism turns out to be, because of killing cell sensitivity it would have to include a stage polarised towards the target cell.

I thank Miss Lynda Strong for technical assistance, and the Medical Research Council, the Imperial Cancer Research Fund and the Institut National de la Santé et de la Recherche Médicale for support. Anti- θ_{AKR} antiserum was given by P. Stern.

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Effects of cytotoxic sera on endothelium *in vitro*

THE rapid rejection of xenografts between widely different species, and the rapid 'hyperacute' rejection of vascularised allografts to previously sensitised recipients are both thought to be due to the effects of cytotoxic antibodies in recipient serum^{1,2}. I have used cultured vascular endothelium to observe the direct effects of cytotoxic sera *in vitro*. Here I examine the effects on pig endothelium of dog serum (as a model for xenograft rejection) and of pig alloantisera to PLA antigens³ (as a model for hyperacute allograft rejection).

Endothelium was obtained from the aortae of five pigs of known PLA type by brief surface digestion with collagenase⁴. Shortly after death the aorta was cannulated by the left subclavian artery and flushed with cold saline. The vessel was dissected out from aortic arch to diaphragm, the intercostal arteries were ligated and a 4 F. G. Portex cannula was tied into each end. It was filled with a warm 0.2 w/v solution of Worthington collagenase (about 500 units ml⁻¹) in tissue culture medium (TCM—Waymouths medium, Burroughs Wellcome, plus 10% heat inactivated foetal calf serum)—and incubated at 37° C for 15-20 min. Detached cells were flushed out with saline, washed with TCM, and plated out in glass 'medical flat' bottles. When required, the cells were resuspended with collagenase and plated at high cell density (150-200 × 10³ ml⁻¹) on to 13 mm diameter glass coverslips in plastic Bijou bottles containing 1 ml of medium. A confluent monolayer was usually obtained in 24 h (Fig. 1a). The coverslips were transferred to 30 mm plastic Petri dishes or to 'Sterilin' slide culture chambers for observation by inverted phase-contrast microscopy. Pigs were tissue typed on the basis of lymphocytotoxic assays using sera raised by skin grafting, kidney grafting or the injection of lymph node cells⁵. Baby rabbit serum was used as a complement source, after testing to exclude intrinsic cytotoxicity. Six different alloantisera were used to produce cytotoxic effects on the endothelial cultures. Experiments were done in duplicate; negative controls of pooled normal serum and compatible alloantisera were included. Fresh dog serum was obtained from three adult mongrels. Heat inactivated dog serum (56° C for 30 min) was used as a control. Pig and dog platelets were prepared from citrated blood by differential centrifugation. The platelet button was allowed to stand for 1 h at 37° C, washed in calcium-free TCM, and resuspended in TCM at a concentration of 500 × 10⁶ ml⁻¹.

Pig kidneys transplanted into dogs show a rapid decline in blood flow within a few minutes of vascularisation. The histological appearances are of almost total obliteration of the smaller vessels by thrombi containing platelets, fibrin and leukocytes. The time course can be slowed by perfusing the kidney with diluted or platelet depleted blood,

or with blood previously passed through a pig liver⁵. Pig endothelium incubated with fresh dog serum showed changes within 15 min in each of four experiments with different endothelial lines, and the monolayer was completely disrupted within 90 min. The first change is that the endothelial cells become more rounded, and draw away from each other so that the cement lines after silver staining, which indicate close intercellular apposition, can no longer be demonstrated. The cells appear granular and vacuolated; many of them extrude cytoplasm as 'ghost bubbles'. At 90 min most of the cells have detached from the substrate and floated away. (Fig. 1b).

The damaged cells become 'sticky' to platelets—resuspended cells treated with dog serum and then mixed with

a dog platelet suspension become surrounded by platelets (Fig. 1c). Dog serum contains a heterophil antibody which agglutinates pig red cells and will cause their lysis in the presence of complement¹. Adsorption of dog serum with pig red cells at 4° C renders it innocuous to pig endothelium, and pig endothelial cells incubated with heat inactivated dog serum will form cross agglutination rosettes with pig red cells.

Exactly comparable effects can be produced by incubating pig endothelium with 'lymphocytotoxic'-alloantisera and then treating it with rabbit serum as a complement source (pig serum is also effective, but some samples are 'anti-complementary'). In five experiments with different endothelial lines, changes were observed after 30 min and were marked at 60 min. Sera toxic to lymphocytes from a given pig were in each case toxic to endothelium from the same pig, but antisera to other specificities, pooled normal serum and specific antibody in the absence of complement were not. The damaged cells were again 'sticky' to pig platelets (these were obtained from pigs compatible with the antiserum used, to avoid the possibility of cross agglutination). The platelet-endothelial cell aggregates are similar to those described by Rafelson *et al.* after treatment of platelet-endothelial cell mixtures with thrombin⁶, and may be related to the platelet thrombi observed *in vivo*.

Similar effects on human endothelium have been observed as a result of treatment with antibodies to HL-A antigens (D. de B. and V. Joysey, in preparation).

I thank Professor R. Y. Calne, Mr M. Slapak, Dr V. Joysey and Mr D. White for help and advice; Mr D. White for providing tissue-typed pigs and pig antisera, and Mrs S. Rogers for providing dog blood. I am grateful to the Medical Research Council of Great Britain for a Junior Research Fellowship.

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Erratum

In the article "Thegosis in herbivorous dinosaurs" by R. A. Thulborn (*Nature*, 250, 729; 1974) the seventh line in para 4 should read 'facets usually occur towards the cervix at the anterior or' . . . and not as printed.

In the article "TRH potentiates behavioural changes following increased brain 5-hydroxytryptamine accumulation in rats" by A. R. Green and D. G. Grahame-Smith (*Nature*, 251, 524; 1974) Figs 1 and 2 were transposed over their appropriate legends.

In the article "B and T-cell stimulatory activities of multiple mitogens from pokeweed" by M. J. Waxdal and T. Y. Basham (*Nature*, 251, 163; 1974) Figs 2 and 3 have been interchanged. The legends are correct as they stand.

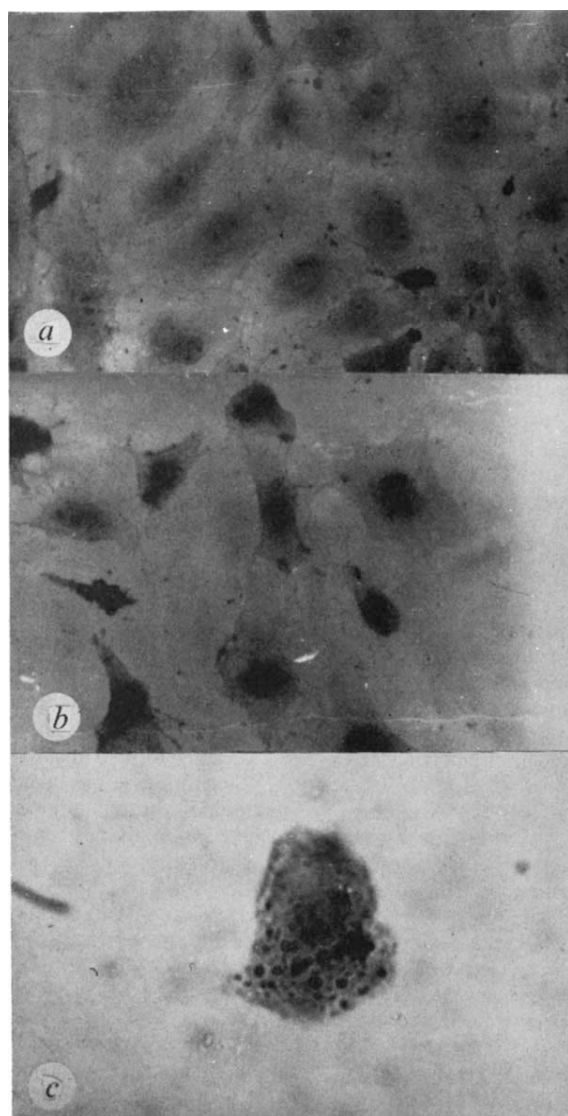


Fig. 1 *a*, Monolayer of normal pig endothelium in culture, stained silver and haematoxylin; *b*, pig endothelium treated for 30 min with 'lymphocytotoxic' antiserum, then for 35 min with baby rabbit serum; *c*, pig endothelial cell surrounded by platelets. About 75,000 resuspended endothelial cells were spun down and treated with 50 μ l of specific pig anti-PLA serum for 30 min, washed, treated with 100 μ l rabbit serum for 30 min and added to 0.5 ml of pig platelet suspension. The mixture was gently shaken for 5 min in a siliconed tube. Smears were made and stained with Giemsa.

matters arising

Taxonomy of the Taung skull

IN discussing the implications of new age estimates for the South African australopithecine cave localities¹, Tobias² suggests if, on further study, it becomes necessary to transfer the Taung skull to the robust lineage, the morphological immaturity of this specimen would preclude its being designated the type specimen for the robust lineage. He further suggests that since this transfer removes the holotype from the paradigm of *Australopithecus africanus*, a lectotype needs to be designated for this species in accordance with Article 74 of the International Code of Zoological Nomenclature. Both of these suggestions are made without basis. The morphological maturity of a specimen or its relative completeness have no bearing on its validity as a holotype. The application of Article 74 is only appropriate when no holotype was designated for a nominal species in the original publication and syntypes exist from which a lectotype can be chosen. Since the Taung skull is still in existence and Dart³ designated no syntypes, Article 74 cannot apply.

It can be appreciated that a renaming of the robust lineage, *A. africanus*, would create confusion for the students of human evolution; however, it is one of the greatest strengths of the Code that it is more difficult to change the validity of a name assigned to a type specimen than it is to change an interpretation based upon the type specimen. Interpretations may change with the seasons but the identity of a type is eternal. The principal purpose of the Code is to produce stability, uniformity and clarity in the way scientific names are applied. In so doing, it does not attempt to regulate the usage of names in reference to species lineages. The Code only concerns the relationship of species names to type specimens and establishes which nominal species have available names and how the type specimen of each is determined. It is the responsibility of the scientist to determine where the type fits in the zoological world and its evolutionary history. The rules of taxonomy and the resulting stability between type specimens and species names do not restrict one's freedom to rearrange specimens in whatever phyletic scheme is warranted by the evidence; it only regulates which names can be validly

applied to the scheme. Tobias's apparent contention that the transfer of the Taung skull to another lineage removes it from being the holotype of *A. africanus* is totally without basis. Transfer of a holotype from one paradigm to another does not affect the association of the holotype with its designated name. The species name remains with the holotype not with the associated paradigm.

A clearer picture of this taxonomic manoeuvring is seen if it is considered that the Taung skull is not being transferred but rather all other specimens previously associated with *A. africanus* are being removed from this paradigm and all specimens assigned to the robust lineage are being transferred to *A. africanus*. Should all the gracile specimens be removed from *A. africanus* the next available name for this group would be *A. transvaalensis* Broom, 1936, the holotype being the Sterkfontein TM 1511 skull⁴. This name has priority over *A. prometheus* Dart, 1948⁵; *Telanthropus capensis* Broom and Robinson, 1949⁶; and *Homo habilis* Leakey, Tobias and Napier, 1964⁷.

This would indeed create a confusing state of affairs and it may seem that following the Code in this case would produce a condition contrary to its stated purpose. The Code (Article 79) does sanction the suppression of a valid species name under extremely exceptional circumstances where the purposes of stability and uniformity are better served by suspending application of the Code. This is the only available procedure for suppressing the usage of *A. africanus* in reference to its type specimen.

Recent years have witnessed a significant improvement in the condition of hominid nomenclature. Three factors have been most responsible for this improvement: first, palaeontologists have ceased regarding every new fossil hominid as a new species; second, palaeontologists, zoologists and physical anthropologists have expanded the concept of variability in palaeospecies making it possible to lump greater ranges of morphological variation within a single species; and, third, the International Code of Zoological Nomenclature has been closely followed by most workers. If hominid nomenclature is to continue to be as parsimonious as possible then a strict adherence to the International Code

of Zoological Nomenclature is mandatory.

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PROFESSOR TOBIAS REPLIES—Olson has done palaeoanthropology a service in showing in what respects I misinterpreted the International Code of Zoological Nomenclature, in the last three sentences of my article¹. Also, he has kindly and lucidly drawn attention to the intricacies of the correct nomenclatural procedures to be used. His comments on Taung are well taken, if one assumes the validity of (1) the original designation of *A. africanus* as the type-species of the new nominal genus *Australopithecus* (Articles 67–70 of the Code), and (2) the basing of the new nominal species on the Taung skull as its holotype (Articles 72–73).

If one assumes the procedures followed were valid, one is left with real problems which rigid adherence to the Code (except for Article 79) would in this instance bring in its wake, as Olson appreciates.

The crux may be stated as follows. The faunal evidence^{2,3}, the estimated date of cave opening⁴ and the sequence of geomorphological events⁵, all point to a young age for Taung. If this age has been correctly inferred, it follows that either (1) Taung may represent a relict population of *A. africanus*; or (2) Taung does not represent *A. africanus* in the sense in which this taxon is understood from Makapansgat and Sterkfontein, a possibility suggested more than 5 years ago⁶. (It does, of course, represent *A. africanus*, the original nominal species.)

If alternative (2) is correct, it would follow that Taung on the one hand, and Sterkfontein and Makapansgat on the other, may represent two different taxa.

In this event, I tentatively raised the possibility that the taxon represented by the Taung skull may be one of the robust taxa (for example, the super-

species, *A. robustus/boisei*). Indeed, fine morphological traits distinguish the Taung teeth from those of Sterkfontein and Makapansgat and, in a few instances, align them with those of the robust australopithecines⁷. If Taung proves to be robust, the confusing position set forth by Olson would follow, namely that the robust australopithecines would need to be added to the hypodigm of *A. africanus*, and the gracile australopithecines from Sterkfontein and Makapansgat would either remain as part of the hypodigm of a vastly more variable *A. africanus*, or need to be removed to form the hypodigm of another species (for which, as Olson points out, the nomen *A. transvaalensis* is available. Since there is now abundant evidence that the gracile and robust australopithecines are taxonomically distinct, the last-mentioned course would probably need to be adopted.

Such removal of the gracile australopithecines of Makapansgat and Sterkfontein from *A. africanus* would cause immense confusion, for the image of the species, *A. africanus*, is based largely on the hominids from these two sites, rather than on the skull of the Taung child, albeit the latter is the holotype.

No less of a muddle would ensue if palaeoanthropologists were now required to call the robust australopithecines *A. africanus*. It was to avert the resulting ambiguity that I raised the possibility of the holotype (the Taung skull) being removed from the paradigm of *A. africanus*—though I assumed and should have added, “if an appeal to the Commission under Article 79 of the Code were successful”. In these unusual circumstances of extreme instability and confusion, not only is it reasonable to expect that such an appeal would be permissible under the Code (as Olson points out), but it might well be justified.

Meantime, the entire discussion has helped to establish the case for a comprehensive restudy of the Taung skull. Professor R. A. Dart, discoverer and nominator of the Taung skull, has generously invited me to undertake such a study.

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Paradox of Earth's core resolved

THE paradox of the Earth's core is hardly resolved by the recent comments of your Geomagnetism Correspondent¹. Even your correspondent states that, because of uncertainties in the values of some of the physical parameters in the Earth's core, it is “not possible to say whether convection in the core is feasible or not”. He also states that “an origin (of the Earth's magnetic field), other than core convection, has never proved viable”, and again “so sure are geomagneticists of the reality of core convection . . .”. It is certainly true that no completely satisfactory explanation has been given for the origin of the Earth's geomagnetic field. Most geophysicists, however, would support the idea that it arises from some kind of dynamo action in the core, but what drives the dynamo is very much a matter of contention. The two most reasonable proposals are thermal convection in the core and the Earth's precession; but in neither case has it been possible to establish that the mechanism would work.

Malkus² quoted experimental work and order of magnitude arguments to suggest that precession may produce turbulent motion in the core and thus drive the geodynamo. Such reasoning can, however, be seriously misleading, and contradictions can often result from over simplification. Malkus³ had earlier suggested that precessional torques may drive the geodynamo. Unfortunately, there are some errors in that article—the detailed theoretical investigation of a dynamo in a precessing turbulent core is extremely difficult and as yet no full treatment has been given. Rochester *et al.* (unpublished work) are working on this problem. At present it is impossible to reject precession as the driving mechanism for the geodynamo—it is as likely as thermal convection in the core.

Finally, your correspondent summarised the “flaw” in the Higgins-Kennedy argument that the core is stable against thermal convection⁴. That, however ‘has already been pointed out (even more forcibly) by me’ both in an earlier letter to *Nature*⁵ and elsewhere⁶.

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Blocking one-way maternal-foetal MLR

THE findings of Jones and Curzen¹ both that lymphocytes from most mothers had low response to stimulating related cord blood lymphocytes in mixed leukocytes reaction, and that only autologous maternal plasmas but not homologous plasmas had an inhibitory effect among those that had relatively high responses, are interesting. Despite the fact that the mixed lymphocyte reaction as measured after 6 d of incubation reflects the sensitisation and responsiveness *in vitro* rather than the state of previous sensitisation *in vivo* as demonstrated by us with the macrophage migration inhibition technique²⁻⁴, the results are consistent with our previous report⁵. It seems that the plasma blocking factors can also inhibit specific sensitisation *in vitro* of maternal cells as well as prevent the expression *in vitro* of cell mediated immune reactivities of lymphocytes previously sensitised (*in vivo*) to solubilised placental antigens as we reported.

On the other hand, the findings of low maternal lymphocyte response to related foetal cells does not warrant the conclusion that maternal lymphocytes may somehow be affected by previous exposure to the plasma blocking factors. The results could as well be accounted for by the abnormality of the foetal cells in their effectiveness as stimulating cells. In fact, if previous exposure had any effect at all, the surface antigens of the foetal cells are a more likely target. The intact responsiveness of the maternal lymphocyte to stimulating related cord or unrelated adult lymphocytes in one-way mixed lymphocyte reaction was indicated by studies by Carr *et al.*⁵.

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